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NUCLEOSIDE TRANSPORT SYSTEMS OF LOW SENSITIVITY TO NITROBENZYLTHIOINOSINE IN CULTURED RAT HEPATOMA CELLS

by

CATHERINE Y. C. NG

(C)

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

DEPARTMENT OF PHARMACOLOGY

EDMONTON, ALBERTA

FALL, 1986

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A R Paterson
.....
Supervisor

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.....

.....

Date 23 May 1986

ABSTRACT

Nitrobenzylthioinosine (NBMPR) is a potent and specific inhibitor of nucleoside transport in various animal cells and erythrocytes. Binding of NBMPR with high affinity to transporter-associated sites has been correlated with inhibition of nucleoside transport in a number of cell types. However, the present study demonstrated that in two lines of cultured rat hepatoma cells, NBMPR occupancy of such sites did not result in inhibition of nucleoside transport.

Cultured cells of the Morris hepatoma 3924A and the Reuber hepatoma H-35 were found to be similar in that high affinity NBMPR binding sites were present, yet adenosine transport in both cell types was of relatively low sensitivity to inhibition by NBMPR (IC_{50} about 1 μM). The NBMPR-binding polypeptide from these cells was of higher molecular weight than similar proteins found in cell types of high NBMPR-sensitivity ($IC_{50} < 10$ nM). Earlier studies in this laboratory showed that NBMPR and several other inhibitors of nucleoside transport increased the tolerance of rodents toward several toxic adenosine analogs. These findings suggested that a "host-protection" tactic using tubercidin-NBMPR-P combinations might be able to exploit in chemotherapy the low NBMPR sensitivity of the nucleoside transport systems of neoplasms. In earlier experiments that tested this tactic, cures of tumor-implanted animals were obtained. The present study attempted to exploit the low sensitivity to NBMPR of the nucleoside transport system of hepatoma 3924A cells by treating rats implanted with these cells with a tubercidin-NBMPR-P combination. While the NBMPR-P component moderated the toxicity of tubercidin, treatment with the combined agents did not reduce tumor growth rates, possibly because of low concentrations of drug in the tumor.

NBMPR binding in 3924A cells was inhibited in the presence of the nucleoside transporter substrates, adenosine and uridine, and the transport inhibitor, dilazep. The inhibition constants obtained were similar to those for the corresponding inhibitions in cells with nucleoside

transporters of high sensitivity to NBMPR. The ability of permeants to inhibit site-specific binding of NBMPR appears to indicate that the NBMPR-binding site in hepatoma 3924A cells is transporter-associated. The relationship between the NBMPR binding site and the permeation site is still uncertain; however, subject to verification in cloned cell lines, the present study indicates that in the rat hepatoma cells studied so far, the two sites appear to be distinct entities.

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ABBREVIATIONS

Ado	adenosine
araA	9-β-D-arabinofuranosyladenine
araC	9-β-D-arabinofuranosylcytosine
Bmax	maximum number of ligand binding sites per cell as determined from equilibrium binding data by mass law analysis
dilazep	3,4,5-trimethoxybenzoic diester with tetrahydro-1H-1,4-diazepine-1,4(5H)-dipropanol
dipyridamole	2,2',2'',2'''-(4,8-dipiperidinopyrimido[5,4*d*]pyrimidine-2,6-diyldinitrilo)tetraethanol
FCCP	carbonylcyanide *p*-trifluoromethoxyphenylhydrazone
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
IC_{50}	concentration at which 50% inhibition occurs
i.p.	intraperitoneal
Kd	dissociation constant
Ki	inhibition constant
Km	substrate concentration for half-maximal unidirectional flux (analogous to the Michaelis-Menten constant)
LD_{50}	median lethal dose; drug dose required to kill 50% of treated animals
Mr	relative molecular weight
NBMPR	6-[(*p*-nitrobenzyl)thio]-9-β-D-ribofuranosyl purine
NBTGR	2-amino-6-[(*p*-nitrobenzyl)thio]-9-β-D-ribofuranosyl purine
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
s.c.	subcutaneous

SDS	sodium dodecyl sulphate
Tris	tris(hydroxymethyl)aminomethane
TU	tubercidin
Urd	uridine
UV	ultraviolet

I. INTRODUCTION

A. Nucleoside Transport in Animal Cells: An Overview

The present study was conducted in a laboratory with interests of long standing in the cellular metabolism of nucleoside analogs[1] with anticancer activity. Transport across the plasma membrane is an important determinant of the cellular metabolism of drugs of this class. This laboratory has current interests in the use of inhibitors of nucleoside transport to manipulate the *in vivo* disposition of these agents and in this way to influence their toxicology. This is part of the context in which the present study was conducted.

Many analogs of the physiological nucleobases and nucleosides have been synthesized as potential therapeutic agents, and many of these compounds possess biological activity, including cytotoxicity. Nucleoside antibiotics comprise a group of nucleoside derivatives found in nature, some with particularly potent biological activities; these compounds have been reviewed by Suhadolnik (1979). Various synthetic nucleosides possess important antiviral activity (Oberg and Johansson, 1984; Hahn, 1979) and antineoplastic activity (Bloch, 1975; Kufe and Spriggs, 1985).

The passage of nucleosides in either direction across plasma membranes in animal cells is mediated by nucleoside-specific transport elements that are intrinsic components of the membrane. The concentration dependence and saturability of transmembrane nucleoside fluxes, and the inhibition of such fluxes by other nucleosides and by specific inhibitors of nucleoside transport indicate that these fluxes are carrier-mediated. Nucleoside transport in

---

[1] The terms, nucleoside analog, nucleoside drug, and unphysiological nucleoside, will refer to pentofuranosyl derivatives of (i) purines and pyrimidines bearing unphysiological groups, (ii) heterocyclic molecules that are analogs of the purine and pyrimidine ring systems, and (iii) various heterocyclic structures unrelated to the nucleobases.

animal cells has been reviewed recently by Plagemann and Wohlhueter (1980), Wohlhueter and Plagemann (1980), Paterson *et al.*, (1981a), Paterson and Cass (1986), and Young and Jarvis, (1983, 1985).

Nucleoside transport systems in human red cells and certain cultured cells appear to be of broad substrate specificity because ribosides and deoxyribosides of the physiological purines and pyrimidines, as well as many unphysiological nucleosides, are accepted as substrates. Km values for the zero-*trans*[2] influx of adenosine and deoxyadenosine are low relative to other physiological nucleosides studied. On this basis, Paterson and Cass (1986) suggested that the adenine nucleosides appeared to be "preferred" substrates for the nucleoside transport system.

Zero-*trans* efflux studies using cells with impaired nucleoside metabolism (thus allowing experimental manipulation of intracellular levels of free nucleosides) have shown that, in some cell types, nucleoside transport is a reversible, non-concentrative facilitated diffusion process inhibitable by nitrobenzylthioinosine (NBMPR)[3] (Plagemann and Wohlhueter, 1980). Under these conditions, the kinetic behaviour of nucleoside fluxes in both directions across the plasma membrane appears to agree with the predictions of the "simple carrier" model of Lieb and Stein (1974), as shown by Plagemann and Wohlhueter (1980).

The potent, tightly bound inhibitors of nucleoside transport recognized by Paterson and co-workers (Oliver and Paterson, 1971; Paul *et al.*, 1975) have been valuable in characterizing transporter biology and function. The best-studied member of the pentofuranoside inhibitors is NBMPR, which binds with high affinity (Kd, 0.1 - 1.0 nM) to plasma membrane sites associated with nucleoside transporters in many cell types. In human erythrocytes and in cultured S49 mouse lymphoma cells (both cell types possess nucleoside transport systems of "high" NBMPR sensitivity[4] ($IC_{50} < 10$ nM)), NBMPR binding at these sites correlates with

---

[2] The zero *trans* notation (Lieb, 1982) refers to the passage of permeant from one side of the plasma membrane, the *cis* side, where the permeant concentration may be varied, to the opposite or *trans* side where the permeant concentration is initially zero.

[3] Abbreviations are defined on pp. ix.

[4] Throughout this presentation, nucleoside transport systems of "high" NBMPR sensitivity will refer to those in which IC_{50} values were 10 nM or less, and "low" NBMPR sensitivity will refer to systems in which IC_{50} values for NBMPR inhibition

inhibiton of transport (Cass *et al.*, 1974, 1981). Recently, nucleoside transport mechanisms of several types, distinguishable on the basis of their interactions with NBMPR, have been recognized in cultured neoplastic cells.

It is an aspect of the low substrate specificity of nucleoside transport mechanisms that transporters are able to accomodate considerable structural variation in the aglycone portion of their substrates, and, accordingly, many unphysiological nucleosides are transporter substrates. Of course, the affinity constants (Km) of these substrates in the transport process may differ with the cell type and from one compound to another, and may be quite different from related physiological nucleosides. Passive diffusion appears to have a minor role in the permeation of cytotoxic nucleoside analogs because (i) AE_1 cells, a mutant clone of S49 mouse lymphoma cells that lack nucleoside transport activity, are resistant to growth inhibtion by cytotoxic nucleosides present in the culture medium (Cass *et al.*, 1979; Cass *et al.*, 1981), and (ii) certain lines of cultured cells are protected against otherwise cytotoxic concentrations of analogs by the presence of transport inhibitors in the growth medium (Paterson *et al.*, 1979a,b). Thus, it is apparent that mediated entry, that is, transport, of nucleoside analogs into cells is a determinant of their biological activity.

B. Facilitated Diffusion

As mentioned above, nucleoside transport has been recognized in certain cell types as a facilitated diffusion process. In this reversible, passive process, net fluxes are dependent upon substrate concentration gradients across the plasma membrane. Rates of these transporter-mediated processes are a function of substrate concentration and are saturable. Transport of a particular nucleoside may be inhibited by other nucleosides and, as well, by various specific inhibitors of nucleoside transport (Paterson *et al.*, 1981a,

---

[4](cont'd) were 1 μM or higher.

1981b). These characteristics demonstrate the existence of transporter-mediated permeation processes, as opposed to permeation by simple diffusion (Stein, 1967).

Facilitated diffusion systems of low specificity mediate nucleoside permeation in many cell types. Transporter specificity has been regarded as "broad" because physiological nucleosides (both ribosides and deoxyribosides) and nucleoside analogues are transported. The broad specificity of the nucleoside transporter of S49 mouse lymphoma cells was clearly shown in comparisons of the cytotoxicity and cellular metabolism of various nucleoside analogs in parental S49 and AE_1 cells, the latter being an S49-derived clone probably bearing a single mutation in the nucleoside transport system (Cohen *et al.*, 1979, Cass *et al.* 1981). The mutation imparted resistance to various cytotoxic nucleosides and impaired the ability of intact AE_1 cells to anabolize these agents, even though the necessary enzymic activities were present in the resistant cells. Of the physiological nucleosides (that is, nucleosides that cells encounter *in vivo*), the adenine nucleosides, adenosine and deoxyadenosine, appear to be "preferred" substrates for nucleoside transport systems with broad substrate specificity. This inference derives from the observation that Km values obtained for adenosine and deoxyadenosine were relatively low compared to those of the other nucleosides studied (Paterson and Cass, 1986).

C. Sodium-Dependent Nucleoside Transport

As noted above, nucleoside permeation via facilitated diffusion systems, which has been reported for many cell types, is non-concentrative and net cellular uptake requires a permeant concentration gradient. In contrast, evidence has been reported for the existence of sodium-dependent nucleoside transport systems (Angielski *et al.*, 1983; Le Hir and Dubach, 1984, 1985; Spector, 1985; Mehra, 1985). Sodium-dependent nucleoside transport in epithelial cells from several tissues has been shown to be concentrative, evidently utilizing the energy inherent in sodium gradients across the plasma membranes. Sodium-dependent nucleoside

transport has been demonstrated in luminal epithelia (Le Hir and Dubach, 1985; Mehra, 1985). The distribution of pumps, channels, receptors and membrane enzymes between the apical and basolateral domains of the plasma membranes of endothelial cells is asymmetrical (Salas *et al.*, 1985).

Nucleoside transport systems that utilize the energy inherent in sodium gradients have been reported to occur in cells of the choroid plexus (Spector, 1985), intestinal lumen cells of the guinea pig (Mehra, 1985) and rat kidney brush border vesicles (Le Hir and Dubach, 1984, 1985; Angielski *et al.*, 1983). Spector (1985) described a high affinity, sodium-dependent transport of nucleosides from the blood into the cerebral spinal fluid in the cells of the choroid plexus in rabbits; the Km for uridine transport in this system was less than 10 μM. In addition, a facilitated diffusion system exists for the transport of nucleosides out of the choroid plexus (efflux) (Spector and Huntoon, 1982) into the extracellular space of the brain. NBMPR is an inhibitor of the efflux process (facilitated diffusion) in this tissue, but not of the influx process (active transport) (Spector and Huntoon, 1982; Spector, 1985).

Uridine transport was shown to be dependent on a sodium gradient in the intestinal brush border vesicles from guinea pig (Mehra, 1985). It is possible that this sodium-dependent system is part of a vectorial nucleoside transport system (i.e. active transport at the apical aspect and passive transport at the basolateral aspect of columnar enterocytes) analogous to that known to transport glucose across the intestinal epithelium (Crane and Dorando, 1982). Active transport (Km, 20 μM) of uridine in these vesicles is of low sensitivity to NBMPR (Mehra, 1985).

Transport of purine and pyrimidine nucleosides occurs by an active process in renal brush border vesicles prepared from rat kidney (Le Hir and Dubach, 1984, 1985). This nucleoside transport system is of low NBMPR sensitivity and appears to utilize the energy inherent in sodium gradients. This process is not dependent on H^+ gradients since FCCP (an H^+ ionophore) did not inhibit the transport activity.

At this early stage in the exploration of energy-dependent nucleoside transport systems, it

appears that these systems may have high substrate affinity (Km $< 10\ \mu M$) relative to the facilitated diffusion systems[5] and that energy-dependent transport may play an important role in the vectorial movement of nucleosides across epithelial cell layers.

Most studies of nucleoside permeation kinetics done prior to 1978 did not measure initial rates of nucleoside uptake. Simple measurements of cellular nucleoside uptake rates were frequently interpreted (incorrectly) to be rates of transport because of the assumption that transport was the slowest event of the cellular uptake process. In this report, "uptake" refers to the appearance of permeant nucleosides and their metabolic products intracellularly while "transport" refers to the carrier-mediated passage of nucleosides across the plasma membrane of cells (Harley *et al.*, 1982; Paterson *et al.*, 1984). The molecular events of the nucleoside transport process are independent of intracellular enzymatic events, such as phosphorylation and deamination. In this sense, nucleoside transport is the initial step in the uptake process.

In cells that metabolize permeants, time courses of permeant uptake are determined by complex factors, as the permeation step is both reversible and independent of metabolism, and, under some conditions, may be faster than subsequent metabolic steps. However, rates of inward transport of nucleoside permeants may be determined from time courses of the cellular accumulation of labelled permeants, even if permeants are substrates for intracellular enzymatic events, because initial rates of cellular uptake of nucleosides are rates of transport, the first step of the uptake process (Paterson *et al.*, 1981a). Because nucleoside fluxes are so rapid in animal cells, the development of rapid sampling techniques was required in order to obtain definitive time courses of nucleoside uptake (Oliver and Paterson, 1971; Wohlhueter *et al.*, 1978a; Paterson *et al.*, 1984).

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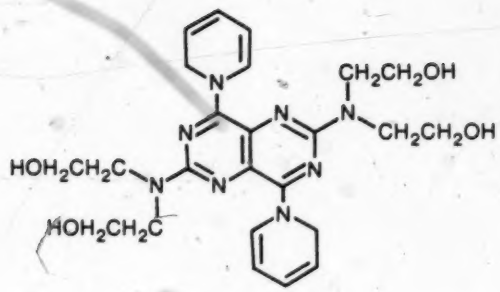
[5] Mean Km values of 20-35 μM for adenosine influx at 22-24° in several cell types have been reported by Paterson *et al.*, (1985). Plagemann and Wohlhueter (1980) reported Km values for adenosine and uridine influx in various cell types that were about 10-fold higher.

D. Inhibitors of Nucleoside Transport

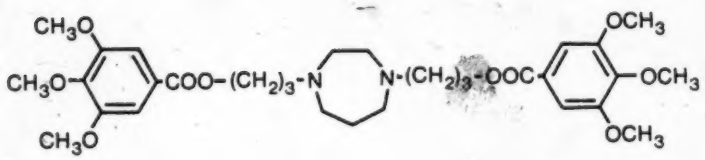
Inhibitors of nucleoside tranport have proved to be invaluable tools in the characterization of nucleoside transporter function, biology and structure. One of the most potent and best studied inhibitors of nucleoside transport is NBMPR (Paterson *et al.*, 1985), an S-substituted 6-thiopurine nucleoside, the structure of which is shown in Figure 1. NBMPR is bound with high affinity (Kd = 0.1 - 1.0 nM) to transporter-associated sites on cells of various types. Cass *et al.* (1974) have shown that the binding of NBMPR to such sites correlated with inhibition of uridine transport in human erythrocytes and in cultured S49 mouse lymphoma cells (Cass *et al.*, 1974, 1981). NBMPR present in the medium of cultured cells protected the cells against the toxic effects of various nucleoside analogs (Paterson *et al.*, 1979; Cass *et al.*, 1981). Certain nucleoside transport systems of low NBMPR sensitivity[4] are discussed later.

E. Non-nucleoside Inhibitors of Nucleoside Transport

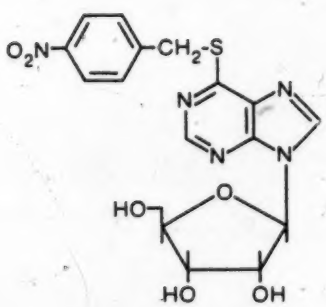
A group of compounds with no obvious structural resemblance to NBMPR have been recognized as potent inhibitors of nucleoside transport and NBMPR binding; members of this group include dipyridamole, dilazep, hexobendine, lidoflazine and papaverine (Van Belle, 1970; Pohl and Brock, 1974; Kolassa *et al.*, 1978; Lum *et al.*, 1979; Paterson *et al.*, 1980, 1983a,b; Plagemann and Wohlhueter, 1980; Marzilli *et al.*, 1984). Of these agents, which were originally developed as vasodilators, dipyridamole is in current use as an antithrombotic agent (Gilman *et al.*, 1985). These agents have been recognized as potent inhibitors of adenosine permeation into cells. It has been hypothesized that the vasodilatory action of dipyridamole derives from blockage of the uptake of plasma adenosine into vascular endothelial cells with a subsequent increase in the availability of extracellular adenosine in the vicinity of adenosine receptors on vascular smooth muscle cells (Kolassa *et al.*, 1978; Klabunde, 1983a,b). Lauzon (1979)



Dipyridamole



Dilazep



Nitrobenzylthioinosine (NBMPR)

Figure 1. Structural formulae of three potent inhibitors of nucleoside transport.

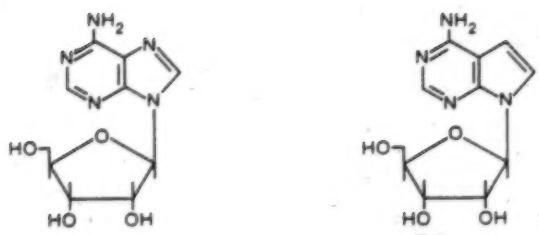


Figure 2. Structural formulae of adenosine and tubercidin.

observed marked inhibition of araC efflux from RPMI 6410 cells treated with dipyridamole.

Other inhibitors of nucleoside transport include thiol reagents (e.g. N-ethylmaleimide, *p*-chloromercuribenzene sulphonate (*p*-CMBS)), cytochalasin B and VP-16 (Plagemann and Wohlhueter, 1980). These compounds may inhibit nucleoside transport by interacting with components of nucleoside transport systems that are different from those with which NBMPR and congeners interact. Inhibition of uridine transport by *p*-CMBS and other thiol reagents implicates thiol groups in the transport process (Dahlig-Harley *et al.*, 1981; Eilam and Cabantchik, 1977; Plagemann *et al.*, 1978).

F. Nucleoside Transporters of High NBMPR Sensitivity

Three types of nucleoside transport systems in cells may be distinguished by their interactions with NBMPR. Nucleoside transporters of high NBMPR sensitivity[4] are found in some cell types in association with polypeptides that bind NBMPR with high affinity. NBMPR occupancy of these binding sites correlates with inhibition of nucleoside transport in human erythrocytes and S49 cells (Cass *et al.*, 1974, 1981). This type of nucleoside transporter appears to be distinct from transporters of low NBMPR sensitivity[4] which have been found in several cell types (Novikoff hepatoma N1S1-67, Walker 256 carcinosarcoma and others discussed below). The transporters of low NBMPR sensitivity may be typed on the basis of their occurence in association with polypeptides (presumably of the plasma membrane) that bind NBMPR with high affinity. Representing one such type is the nucleoside transport system of Walker 256 cells which are devoid of high affinity NBMPR binding sites (Kd, 0.1 to 1.0 nM) and possess nucleoside transporters of low NBMPR sensitivity. Representing nucleoside transporters of a different type, are those systems of low NBMPR sensitivity found in cells that possess high affinity NBMPR binding sites (Novikoff hepatoma cells and others discussed below).

Cells with transporters of high NBMPR sensitivity include S49 mouse lymphoma cells (Cass *et al.*, 1981), RPMI 6410 cells (Paterson *et al.*, 1979), and human erythrocytes (Cass *et al.*, 1974; Jarvis and Young, 1980). In the presence of NBMPR, S49 mouse lymphoma cells proliferating in culture were protected against lethal concentrations of tubercidin, a cytotoxic analog of adenosine, apparently because NBMPR rendered the cells impermeable to the analog. S49 cells possess high affinity sites that bind NBMPR (Kd about 0.1 nM) and uridine transport is blocked by concentrations of NBMPR less than 100 nM (Cass *et al.*, 1981). The NBMPR binding polypeptide from S49 cells migrated with a Mr of 45,000-66,000 in polyacrylamide gel electropherograms (Young *et al.*, 1984).

Cells of the AE_1 clone, derived by mutation from S49 cells, are deficient in nucleoside transport activity. This clone was selected for adenosine resistance from chemically mutagenized cells of the S49 parent line (Cohen *et al.*, 1979).

G. Nucleoside Transporters of Low NBMPR Sensitivity

Cell types that possess nucleoside transport systems of low NBMPR sensitivity include cultured cell lines of the Novikoff UA hepatoma and the Walker 256 rat carcinosarcoma. Micromolar concentrations of NBMPR must be present extracellularly before significant inhibition of transporter function in these cells is observed. As noted below, this insensitivity to NBMPR does not correlate with the absence of sites that bind NBMPR.

Cells of the Novikoff UA line differ from those of the parental line (Novikoff N1S1-67) in that NBMPR binding sites were demonstrated in the former (Gati *et al.*, 1986), but were not found in the latter (Plagemann and Wohlhueter, 1984a).[6] The nucleoside transport systems

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[6] The Novikoff N1S1-67 cell line used by P. G. W. Plagemann and coworkers in studies reported prior to 1984 was uncloned (P. G. W. Plagemann, personal communication) and contained variant cell types. N1S1-67 cells from the Plagemann laboratory were passaged in this laboratory with adaptation to MEM medium containing 10% horse serum and, after 1 year of passage, the line was designated Novikoff UA. The culture conditions evidently selected cells of a

of both cell lines were shown to be of low sensitivity to NBMPR (Plagemann and Wohlhueter, 1984a; Gati *et al.*, 1986). The affinity (Km) for uridine of the nucleoside transport system in Novikoff UA rat hepatoma cells is within the range reported for other cell types (Jakobs and Paterson, 1984). Cloned Novikoff UA cells demonstrate wide heterogeneity in expression of high affinity NBMPR binding site proteins. Clones with and without NBMPR binding sites have been isolated (without mutagenesis) from the Novikoff UA cell line (J. A. Belt, personal communication). Also, the NBMPR-binding polypeptide of Novikoff UA cells is of higher Mr (72,000 - 80,000) than that of S49 cells (Gati *et al.*, 1986).

The kinetic characteristics of nucleoside transport in cultured Novikoff hepatoma cells and Walker 256 carcinosarcoma cells, cells with transporters of low NBMPR sensitivity (Plagemann and Wohlhueter, 1980; Belt, 1983), are generally similar to those in cells with transporter systems of high NBMPR sensitivity.

H. Cells with Nucleoside Transporter Systems of High and Low Sensitivity to NBMPR

A number of cell types have high affinity binding sites for NBMPR, but, unlike human erythrocytes and S49 cells, total occupancy of these sites by NBMPR does not cause total inhibtion of nucleoside transport. For example, Lauzon and Paterson (1977) demonstrated that (i) in the presence of 5 nM NBMPR, high affinity binding sites were occupied, yet a substantial fraction of nucleoside transporter activity remained, and (ii) a 1000-fold increase in NBMPR concentration abolished transport of uridine and thymidine. Dahlig-Harley *et al.*, (1981) presented a clear example of a biphasic concentration-effect plot for NBMPR inhibition of uridine transport in HeLa cells.

A similar biphasic concentration-effect relationship for the NBMPR inhibition of uridine

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'(cont'd) genotype in which the NBMPR binding protein was expressed, and cells of this type became more abundant in the Novikoff UA cell line than in the N1S1-67 line.

transport in a cloned line of mouse leukemia L1210 cells was interpreted by Belt (1983) to mean that the leukemic cells possess nucleoside transport systems of two types, one with high sensitivity to NBMPR and the other with low sensitivity to NBMPR. Plagemann and Wohlhueter (1984a) have also reported biphasic relationships for the NBMPR inhibition of uridine transport in several cell types, including CHO and P388 cells, and have speculated that, while the relationships between the two types of nucleoside transport systems are presently unknown, they may be two forms of the same system.

I. Use of Inhibitors in Studies of Nucleoside Transporter Function and Biology

Use of NBMPR as a probe has led to a greater understanding of the biology and function of nucleoside transport systems, as the following examples indicate.

a. Enumeration of NBMPR Binding Sites

A substantial effort has been spent in exploration of the relationships between NBMPR occupancy of high affinity binding sites and NBMPR effects on transport. Initially, studies with erythrocytes, cells in which nucleoside transport is entirely sensitive to NBMPR, led to the view that the high affinity sites to which NBMPR was bound represented nucleoside transport elements and, if the assumption was made that each transporter bound a single NBMPR molecule, transporter capacities could be calculated. Major support for these ideas came from the following work:

(i) Cass *et al.* (1974) showed that fractional inhibition of uridine transport in human erythrocytes was proportional to fractional occupancy of binding sites by NBMPR.

(ii) AE_1 cells, a mutant clone of S49 cells, possessed no detectable NBMPR binding sites, and were resistant to the antiproliferative effects of cytotoxic nucleosides (Cass *et al.*, 1981).

(iii) Jarvis and Young (1980, 1982a) demonstrated that erythrocytes from sheep of a particular allelotype (Nu^r,Nu^r) possessed high affinity NBMPR binding sites, but cells from sheep of another allelotype (Nu^r,Nu^-) lacked uridine transport capability and also, lacked the binding sites.

(iv) Jarvis *et al.* (1982) demonstrated that the abundance of NBMPR binding sites in erythrocytes from different species correlated with the uridine transport capacity (Vmax) of these cells. They concluded that species differences in the nucleoside transport capacity of erythrocytes were due primarily to differences in the cellular number of functional nucleoside transporters rather than to species differences in the kinetic properties of the transporters. With the assumption that each molecule of bound NBMPR represented a functional transporter, the uridine transport capacity of transporters from erythorcytes from a number of species was remarkably similar.

These observations support the idea that the presence of high-affinity binding sites for NBMPR represent functional, nucleoside transport elements of high NBMPR sensitivity.

High-affinity NBMPR binding has been reported in synaptosomes (Marangos *et al.*, 1982) and in crude membrane preparations from guinea pig heart (Hammond and Clanachan, 1983), rat lung (Shi *et al.*, 1984) and heptocytes (Wu and Young, 1984). Jarvis *et al.* (1982) showed that NBMPR binding sites were present on erythrocytes of the human, rabbit, mouse, rat and guinea pig, but not on those of the dog.

As exploration of the relationships between NBMPR binding site occupancy and nucleoside transport inhibition in cultured cell systems has progressed, it has become apparent that (i) those relationships are complex in some cell types, and (ii) sites that bind NBMPR with high affinity do not necessarily represent functional nucleoside transporters (as this study shows). However, in some cell types, the converse is probably true, that is, NBMPR binding sites are associated with transporters of high NBMPR sensitivity ($IC_{50} < 10$ nM).

b. Proteins Involved in Nucleoside Transport

Photoaffinity labelling studies with ^{3}H-NBMPR as a photoactivable ligand have enabled electrophoretic identification of NBMPR binding proteins in cell fractions that contain plasma membranes. Upon exposure to UV light, a covalent bond is formed between site-bound NBMPR and polypeptides comprising the site. NBMPR binding sites in crude membrane preparations from human erythrocytes (Wu *et al.*, 1983), S49 cells (Young *et al.*, 1984), and from several guinea pig and rat tissues (Shi *et al.*, 1984; Wu and Young, 1984) have been photolabelled in this way. The photolabelled membrane preparations were solubilized with sodium dodecyl sulphate and subjected to polyacrylamide gel electrophoresis using the buffers described by Laemmli (1970). The binding protein, labelled by covalently bound [G-^{3}H]NBMPR, was found as a band migrating with a Mr of 45,000 to 66,000 in membrane preparations from S49 cells (Young *et al.*, 1984) and from human red blood cells (Wu *et al.*, 1983).

Because of the above-noted relationships between NBMPR occupancy of the high affinity sites and inhibition of nucleoside transport in several cell types, the NBMPR binding polypeptide has been regarded as an integral part of the nucleoside transporter in human erythrocytes (Young *et al.*, 1983). However, the subsequent recognition of nucleoside transport systems of low NBMPR sensitivity[4] and the coexistence of the latter with NBMPR binding proteins in particular cell types, indicate that (i) NBMPR-binding polypeptides should be regarded more properly as "transporter-associated" in some cell types, and (ii) the polypeptides directly involved in permeant translocation have yet to be conclusively identified. The present study bears on this issue. Radiation inactivation studies with human erythrocytes have indicated that both nucleoside transport and the NBMPR binding activities are inactivated with similar molecular weights of about 122,000 (Jarvis *et al.*, 1980; Jarvis *et al.*,1984).

c. Transport Inhibitor as "Stoppers" in Kinetic Studies of Nucleoside Permeation

As noted above, initial rates of cellular uptake of nucleosides define inward transport rates. For time courses of nucleoside uptake to be definitive of initial rates, it has been found that initial intervals of uptake must be very brief, that is, in the order of 2-5 sec. Inhibitors of nucleoside transport have been used as "stoppers" to rapidly end intervals of cellular uptake of nucleosides. Of course, this use of inhibitors is only valid in cells with nucleoside transport systems in cells that are demonstrably sensitive to the inhibitors under consideration. NBMPR and congeners have been employed for this purpose in the development of rapid sampling methods for measuring rates of nuclesoide transport in erythrocytes (Pickard *et al.*, 1972; Cabantchik and Ginsburg, 1977) and in S49 cells (Cass *et al.*, 1981; Paterson *et al.*, 1983a), HeLa cells and L5178Y cells (Harley *et al.*, 1982).

Cass *et al.* (1981) employed 5 μM NBMPR to end intervals of adenosine uptake in S49 cells. Similarly, Harley *et al.* (1982) demonstrated nearly "instantaneous" inhibition of adenosine transport by HeLa cells and mouse lymphoma L5178Y cells by rapid addition of NBMPR to a final concentration of 8 μM. A quenched-flow system, using 333 μM dilazep as a quencher of the transport process, has allowed measurement of adenosine transport during decisecond intervals in S49 cells (Paterson *et al.*, 1984). Time courses of permeant uptake by cells between 0.05 and 0.5 sec after initiation of uptake were approximately linear in the latter system. A particularly important aspect of the use of inhibitors as transport stoppers is that time courses may be started from time zero. Values for the permeant content of cells at time zero may be obtained simply by exposing cells to the stopper before the permeant. Time-zero values are not directly obtainable with the widely used oil-layer centrifugation procedure for ending intervals of permeant uptake by cells.

Thus, inhibitors of nucleoside transport have played an important role in exploration of the nucleoside transport process by means of kinetic studies.

J. The Nucleoside Permeants Employed in this Study

Adenosine and its analog, tubercidin (Figure 2), were employed as permeants in this study. An earlier kinetic study from this laboratory indicated that both nucleosides were equivalent transporter substrates, being transported inwardly with similar kinetic constants in several cell types (Harley *et al.*, 1982).

a. Adenosine

Adenosine is involved in the regulation of lipolysis, platelet aggregation (Newby, 1984), neural functions (Phillis and Wu, 1981; Wu and Phillis, 1984), and lymphocytic differentiation (Polmar *et al.*, 1983). For reviews, see Daly (1983) and Breckenridge, (1983). This compound exerts its regulatory actions through receptors, thought to be located in the extracellular surface of the plasma membrane. It has been hypothesized that (i) mechanisms for the clearance of adenosine from the vicinity of the receptors are necessary in adenosine regulatory systems, and (ii) cellular uptake and metabolism of adenosine may serve this purpose. Since transport is the first step in the cellular utilization of adenosine, it has been postulated that inhibition of adenosine transport by dipyridamole, dilazep, NBMPR, and other vasodilatory agents would tend to increase the extracelullar concentration of adenosine and thereby potentiate its effects (Pohl and Brock, 1974; Kolassa *et al.*, 1978; Lum *et al.*, 1979; Paterson *et al.*, 1980, 1984; Berne *et al.*, 1983; Marzilli *et al.*, 1984).

b. Tubercidin

Some cytotoxic nucleosides have antineoplastic activity. Examples include araC (Cytarabine), araA (Vidarabine), 6-thioguanosine, 5-azacytidine, tubercidin and showdomycin. Because of the wide substrate specificity of the nucleoside transporter, these

drugs are readily transported into cells and enter cellular metabolic processes. Interference with protein synthesis or incorporation into cellular DNA and RNA are among the means by which metabolites of these agents may exert their cytotoxic effects. For example, tubercidin (Figure 2), an antibiotic produced by *Streptomyces tubercidus* (Suhadolnik, 1970, 1979), is transported into cells and subsequently phosphorylated by adenosine kinase (Parks and Brown, 1973). In cultured L5178Y cells, tubercidin triphosphate has been shown to inhibit mRNA synthesis by competing with physiological nucleoside triphosphates in enzymatic reactions (Müller, 1979). Other effects include interference with energy metabolism, inhibition of *de novo* purine synthesis, and the processing of rRNA (Müller, 1979). The cytotoxic effects of tubercidin evidently derive from such biochemical actions.

K. The Host-Protection Tactic: Use of NBMPR in Combination with Cytotoxic Nucleosides

Cultured cells of several types are protected in the presence of NBMPR from the toxic effects of nucleosides. Proceeding from this observation, Lynch *et al.* (1981a) and Paterson *et al.* (1979b) demonstrated protection of mice by NBMPR and its 5'-phosphate (NBMPR-P)[7] from potentially lethal doses of nebularine and tubercidin. Lethal doses of these agents, given together with host-protecting doses of NBMPR-P, were then employed in the treatment of mice implanted with leukemia L1210. Treatment with the NBMPR-P-nebularine combinations achieved substantial kill of the leukemic cells, demonstrating that nebularine-sensitive tissues of the host were better protected than the leukemic cells (Lynch *et al.*, 1981a). Similarly, selective toxicity toward three other transplantable tumor cell lines (Co26 carcinoma, the Ehrlich ascites carcinoma, and leukemia L1210/TG8) resulted from treatment with tubercidin-NBMPR-P combinations (Lynch *et al.*, 1981b).

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[7] NBMPR-P, a prodrug form of NBMPR, has greater aqueous solubility than the parent compound. NBMPR-P has no nucleoside transport inhibitory activity, but, *in vivo*, NBMPR is released from NBMPR-P by ecto 5'-nucleotidase activity (Ogbunude *et al.*, 1984).

The experiments described above employed optimized NBMPR-P dosages, since supraoptimal dosages protected certain neoplastic cells, as well as host tissues (Lynch *et al.*, 1981b). Against that background, the idea was then tested that neoplastic cells with nucleoside transporters of low NBMPR sensitivity would be responsive to chemotherapy with high doses of nucleoside analogs given with host-protective doses of NBMPR-P. Jakobs and Paterson (1984) employed the Walker 256 rat carcinosarcoma for this purpose because cultured cells of that line lack high affinity binding sites for NBMPR, and, consequently, nucleoside transport in those cells was relatively insensitive to the inhibitor. Their study showed that (i) male Sprague-Dawley rats were protected against otherwise lethal doses of tubercidin by NBMPR-P, and (ii) treatment of rats implanted with the Walker 256 carcinosarcoma with the tubercidin-NBMPR-P combination "cured" a high proportion of the animals.

II. INTRODUCTION TO THE PRESENT STUDY

The objective of the present study was to characterize nucleoside transport systems in transplantable rodent cancer cells that would serve as models in further tests of the host protection tactic in chemotherapy (see Section I.K). The Morris rat hepatoma 3924A was selected for study in the present project because (i) this neoplasm may be propagated either *in vitro* or *in vivo*, and (ii) the report of Zhen *et al.* (1983) indicated that nucleoside salvage in 3924A cells was impaired by dipyridamole, but not by NBMPR.

In the present study, the presence of a nucleoside transport system of low NBMPR sensitivity was demonstrated in cultured hepatoma 3924A cells, suggesting that the *in vivo* line of this neoplasm might be an appropriate model for testing the host protection tactic. Characterization of nucleoside transport in 3924A cells showed that these cells possessed both (i) sites that bound NBMPR with high affinity, and (ii) a nucleoside transport system of low NBMPR sensitivity[4]. The apparent association of these two characteristics is surprising, and, if validated, may have important implications with respect to transporter structure. Similar characteristics were also found in a cultured line of the Reuber H-35 rat hepatoma cells.

Because of the low sensitivity of the nucleoside transport system of 3924A hepatoma cells, an attempt was made to test the host protection tactic in therapeutic experiments with the *in vivo*-passaged line of this neoplasm in ACI/N rats, the strain of origin. Tubercidin was selected as the therapeutic agent and treatment regimens were established for the administration to ACI/N rats of high, potentially lethal tubercidin doses together with protecting doses of NBMPR-P. A single experiment was undertaken in which rats bearing subcutaneous implants of hepatoma 3924A were treated with the tubercidin-NBMPR-P combination by the intraperitoneal route. The treatment failed to change the growth kinetics of the subcutaneous tumors, possibly because of poor access of the therapeutic agent to the neoplastic cells.

III. MATERIALS AND METHODS

Cultures of an *in vitro* line of the Morris rat hepatoma 3924A were generously provided by Dr. George Weber (Laboratory of Experimental Oncology, Indiana University School of Medicine, Indianapolis, IN). Stock cultures were grown as monolayers in 80 cm^2 flasks (Nunc, Roskilde, Denmark) with McCoy's 5A medium supplemented with 10% fetal calf serum in a 5% CO_2-air atmosphere at 37° in a humidified incubator. Under these conditions, cell population doubling times were about 15 hours. Frozen stocks of Reuber H-35 hepatoma cells were obtained from the American Type Culture Collection (Rockville, MD) and these cells were adapted to grow in McCoy's 5A medium with 10% fetal calf serum. The mean generation time of this cell line under these conditions was about 20 h. Cultures were free of *Mycoplasma* (Dr. J. A. Robertson, Department of Medical Microbiology, University of Alberta) and stock cultures were restarted from frozen stock at 8 - 12 week intervals. To maintain cultures in exponential growth, stock cultures of hepatoma 3924A cells were subcultured twice weekly, and H-35 cells were subcultured once weekly using innocula of 1.5×10^3 cells/ml for both cell lines. When cells of either line were propagated for purposes other than maintenance of stock cultures, growth medium contained antibiotics (penicillin G, 100 units/ml; streptomycin, 100 μg/ml).

Cell culture materials were obtained from Gibco Laboratories (Burlington, Ontario). [G-^{3}H]NBMPR (37 Ci/mmol) and [2,8-^{3}H]adenosine (23 Ci/mmol) were purchased from Moravek Biochemicals Inc., Brea, CA, and were repurified when necessary by high pressure liquid chromatography to >98% radiochemical purity using a C_{18} μ Bondapak column (Waters Associates, Milford, MA) eluted with methanol/water solutions[9]. NBMPR and NBTGR were prepared in this laboratory by Herta Unger by established methods (Noel and Robins, 1962; Paul *et al.*, 1975) using as starting materials 6-thioinosine and 6-thioguanosine provided by the

[9] Purified ^{3}H-nucleosides were generously provided by Dr. E. S. Jakobs of this laboratory.

Division of Cancer Treatment, National Cancer Institute, Bethseda, MD. NBMPR-P was prepared as the disodium salt (Lynch *et al.*, 1978) by the Research Laboratory of Yamasa Shoyu Ltd., Chosi, Japan. Dipyridamole and dilazep were gifts from Boehringer-Ingleheim (Canada) Ltd. (Dorval, Quebec), and Hoffmann-La Roche and Co. (Basel, Switzerland), respectively. Tubercidin and deoxycoformycin were generously provided by the Division of Cancer Treatment, National Cancer Institute (Bethseda, MD). ACI/N rats were purchased from the Laboratory Supply Company, Indianapolis, Indiana.

A. Cell Proliferation Rates

To measure the effects of nucleoside analogs on proliferation rates of hepatoma cells in culture, replicate cultures were prepared and, after 24-h intervals to establish the monolayers, growth medium was replaced with fresh growth medium containing the drugs under study. To prepare replicate cultures, cells were seeded onto 35-mm diameter vented petri dishes (Nunc, Roskilde, Denmark) at a density of 5×10^4 cells/dish. After 24 h, the medium was replaced by drug-containing medium and at 16-h intervals thereafter, triplicate cultures were assayed for cell number by trypsinization of the monolayers and enumeration with a Model ZF Coulter Counter (Coulter Electronics, Hialeah, FA). Cell proliferation rates were expressed as the number of population doublings that occurred during 24 h of growth.

B. Antiproliferative Effects of Tubercidin

Cells were seeded as described above and, after 24 hours, the medium was replaced with fresh medium containing graded concentrations of tubercidin with or without 5 μM NBMPR (final concentration). After a further 48-h interval of culture, cell numbers in the monolayers were enumerated.

C. Binding Studies with Hepatoma 3924A Cells

Equilibrium binding of [G-^{3}H]NBMPR was used to determine numbers of high affinity NBMPR binding sites on cultured cells of the rat heptomas 3924A and H-35. Total binding was determined from the cellular ^{3}H-content after incubating the monolayers for 20 min at 22° with graded concentrations of [G-^{3}H]NBMPR with and without 5 μM non-isotopic NBMPR (Cass *et al.*, 1981). These experiments employed replicate monolayer cultures with 1 - 1.2 x 10^6 cells per 35-mm plastic culture dish. Intervals of ligand-cell equilibration were ended by removal of the ligand-containing medium by suction from the culture dishes, and monolayers were at once rinsed rapidly 5 times with 2-ml portions of ice-cold Dulbecco's PBS (Dulbecco and Voght, 1974). Cultures were processed individually through the procedures that ended intervals of equilibration. In the washing procedure, culture dishes were placed on an angled, ice-cooled metal tray and the rinse liquid was removed by continuous suction as it flooded over the monolayer (Heichal *et al.*, 1978). After the monolayers were drained by inversion on absorbent paper, 0.75 ml of 5% Triton X-100 (v/v) was added to each culture dish, and, to promote solubilization, the dishes were incubated at 37° in a humidified incubator for 20 min.

To determine ^{3}H-content, 0.5-ml portions of the solubilized samples were mixed with 8 ml of a Triton X-100-xylene scintillation cocktail fluid (Pande, 1976) and assayed for ^{3}H activity by liquid scintillation counting. Binding assays were conducted in triplicate. Concentrations of ^{3}H-NBMPR in incubation mixtures were determined from the initial ^{3}H-content; equilibrium concentrations of ^{3}H-NBMPR were determined by deducting from initial concentrations the total ^{3}H-NBMPR content of each monolayer at equilibrium. Table 1 compares the values of the binding constants obtained using equilibrium concentrations of free NBMPR determined in this manner with constants obtained with direct assays of free NBMPR concentrations at equilibrium.

Table 1. NBMPR binding constants obtained using equilibrium concentrations of NBMPR determined by two methods.

	Measured		Calculated	
	Kd (nM)	Bmax (sites/cell x 10^{-5})	Kd (nM)	Bmax (sites/cell x 10^{-5})
Experiment 1	0.42±0.03*	2.7±0.3*	1.03±0.17	3.1±0.3
Experiment 2	0.60±0.16	3.5±1.2	1.12±0.21	3.3±0.9

* S.D.

D. Inhibition of Site-Specific NBMPR Binding to Hepatoma 3924A Cells

To test the idea that the high affinity NBMPR binding sites on 3924A cells were part of the nucleoside transport system, it was determined whether permeants (adenosine and uridine) affected NBMPR binding and whether dilazep, a potent inhibitor of nucleoside transport, also perturbed NBMPR binding in hepatoma 3924A cells. In a similar experiment with H-35 cells, adenosine only was evaluated as an inhibitor of NBMPR binding. Reciprocal plots of the cellular content of site-bound NBMPR *versus* the equilibrium concentration of NBMPR were used in mass law analysis of NBMPR binding data (Edsall and Wyman, 1958).

E. Km of Adenosine Permeation in Hepatoma 3924A Cells

For the inward transport of adenosine in various cultured cell lines and erythrocytes, mean Km values of 20 to 35 μM have been reported (Paterson and Cass, 1986). A kinetic study of adenosine influx in hepatoma 3924A cells was undertaken in the present work. In characterizing the unusual nucleoside transport system of this cell line, our approach to the assay of adenosine transport rates was to measure initial rates of ^{3}H-adenosine uptake into replicate monolayers from time courses of adenosine uptake. Since adenosine transport is a rapid process, very brief intervals of adenosine uptake were employed in order to define initial uptake rates. Because of the low sensitivity of the nucleoside transport system of 3924A cells, NBMPR or congeners could not be employed as "stopping" agents, as in other studies reported from this laboratory (Harley *et al.*, 1982; Paterson *et al.*, 1984). Intervals of uptake were started by flooding the monolayers with medium containing ^{3}H-adenosine, and were ended by rapid immersion of monolayers into large volumes of ice-cold Dulbecco's PBS. The minimum practical interval of uptake with this procedure was 5 sec. These operations were timed by means of metronome signals.

Replicate monolayers (1 - 1.2 x 10^6 cells/culture) grown in 35-mm culture dishes were

exposed at 22° for intervals of 0, 5, 8, 11, 14, 17 and 20 s to graded concentrations of [2,8-^{3}H]adenosine in "transport medium" (McCoy's medium without serum and carbonate, but supplemented with 20 mM HEPES (pH 7.4)). Intervals of uptake were started by flooding the monolayers with 0.5 ml of adenosine-containing medium and were terminated by rapidly submerging the culture dishes into a 2-litre volume of ice-cold Dulbecco's PBS. Two seconds prior to the first immersion, most of the medium was removed from the culture dish by aspiration. Culture dishes were then rinsed by one immersion in each of two additional 2-litre volumes of ice-cold Dulbecco's PBS. Monolayers were then dissolved in 0.75 ml 5% Triton X-100 (v/v) (with incubation for 20 min at 37° in a humidified atmosphere), and 0.5-ml samples were assayed for radioactivity by liquid scintillation counting.

The cellular uptake of adenosine during an interval of zero seconds (that is, "cellular uptake at time zero") was approximated as follows. Monolayers were exposed for 20 min to transport medium containing 10 mM non-isotopic adenosine at 22°, drained, and then placed on ice for 30 sec. To measure adenosine uptake, monolayers were flooded with 0.5-ml portions of isotopic permeant as in the above procedure and, as soon as possible thereafter, uptake was ended by immersion of the monolayers into ice-cold Dulbecco's PBS solution and rinsed twice as described above. Samples were then assayed for radioactivity. Initial rates of transport were measured from time-zero tangents to cellular uptake plots. All assays were conducted in triplicate.

Corrections for a time-dependent non-mediated uptake or association of adenosine with 3924A cells in addition to the time-zero correction (see above) were not made, as in another study of nucleoside transport in cultured Walker 256 cells, a line of low NBMPR sensitivity (Belt and Noel, 1985). In earlier reports, this laboratory has shown that rates of non-mediated entry of nucleosides into cultured cells are very low (Paterson *et al.*, 1979b; Cass *et al.*, 1981). While the rates of adenosine uptake reported here might have been reduced to some extent by the application of correction for non-mediated entry, the reported rates are probably a close approximation of transport rates.

F. Effect of Nucleoside Transport Inhibitors on Adenosine Transport in Cultured Cells of Hepatoma Lines 3924A and H-35

The effects of NBMPR and other inhibitors of nucleoside transport on adenosine permeation were evaluated by exposing monolayer cultures to the inhibitors for 10 min before assaying adenosine transport rates in the presence of the inhibitors. Replicate monolayer cultures containing 1 - 1.2 x 10^6 cells/dish were incubated with graded concentrations of NBMPR in McCoy's transport medium for 10 min prior to assays of adenosine transport. The concentration of [2,8-^{3}H]adenosine used was 20 μM and intervals of uptake were 5 sec. The cellular content of ^{3}H-adenosine was determined as in Section E, and all assays were conducted in triplicate. The effects of other inhibitors of nucleoside transport (dipyridamole, dilazep and hexobendine) were also evaluated in this way.

G. Photoaffinity Labelling of the NBMPR Binding Protein in Cultured Cells of Hepatoma Lines 3924A and H-35

Stock cultures of hepatoma 3924A cells were expanded to 500 ml in McCoy's 5A medium with 100 units/ml penicillin and 100 μg/ml streptomycin in spinner flasks. These cultures were then used as innocula for suspension cultures in 5-l round bottomed flasks, in which cultures were kept agitated by means of vibrating impellers (Model E1 Vibro-Mixer, Chemapac, Hoboken, NJ) (Paterson *et al.*, 1979). Cells were harvested by centrifugation when cell concentrations were 1 - 2 x 10^6 cells/ml, concentrations at which cells proliferated exponentially. Centrifugally sedimented cells were resuspended in a buffered salts solution (150 mM sodium chloride, 20 mM HEPES (pH 7.4, 4°), 2 mM magnesium chloride, 1 mM EDTA) and were then disrupted by nitrogen cavitation at 400 psi at 8° (Ross *et al.*, 1977) using a Parr cell disruption chamber (Parr Instrument Company, Moline, IL). Disrupted cell preparations were centrifuged (900 x *g* for 5 min at 4°) and the supernatant fractions were then

centrifuged at a higher gravitational force (43,000 x *g* for 20 min at 4°). The final pellets, which contained plasma membranes, were resuspended in 50 mM Tris-HCl (pH 7.4 at 4°) using a Dounce homogenizer. Portions (1.0 ml) of these membrane-containing preparations were then sealed in glass ampoules and stored in liquid nitrogen. Stock cultures of H-35 cells were expanded as monolayers in roller bottles and when cells in the monolayers were confluent (about 10^8 cells per roller bottle (1400 cm^2)), cells were harvested with a plastic scraper and membrane-containing fractions were prepared as just described.

To photolabel high-affinity sites on hepatoma 3924A plasma membranes, 50 - 100 µl portions (15 µg protein/µl) of the membrane preparations were exposed to 50 nM [G-^{3}H]NBMPR in 50 mM Tris-HCl (pH 7.4) with 50 mM dithiothreitol present to serve as a free radical scavenger during UV photoactivation. After incubation for 15 min at 22° in the presence and absence of 10 µM NBTGR as a competing ligand, the reaction mixtures were transferred to silica spectrophotometer cuvettes (1-mm light path) for exposure to UV light for a 1-min interval. The cuvettes were placed 4 cm from the outside of the silica cooling sleeve of a 450-watt mercury arc lamp (Canrad-Hanovia Inc., Newark, NJ) and approximately 5.5 cm from the lamp surface. After UV exposure, samples were then diluted 75-fold with buffer containing 10 µM NBTGR and allowed to equilibrate at 22° for 20 min before recovery of the crude membrane preparation by centrifugation (43,000 x *g* for 20 min). The membrane-containing pellets were finally resuspended in 40 µl Tris-HCl and dissolved by addition of 40 µl of SDS sample buffer (SDS (5% w/v), 5 mM Na_2EDTA, glycerol (10% w/v), bromophenol blue (0.1 mg/ml), 2 mM phenylmethylsulfonyl fluoride, 10 mM dithiothreitol, and 20 mM Tris-HCl (pH 8.0, 22°). Portions (40 µl) of the solubilized preparations were subjected to electrophoresis in 1.5-mm thick slab SDS-polyacrylamide gels by the method of Laemmli (1970). Gel lanes were cut into 2-mm slices with a gel slicer (Model 190, Bio-Rad Laboratories) and the slices were incubated at 37° for 24 h with 10 ml of a 3% solution of Protosol (New England Nuclear, Lachine, Quebec) in Econofluor (New England Nuclear) to solubilize the protein content of the slices. Samples were left in the dark at room temperature

for 48 h to reduce chemiluminescence prior to assay of ^3H by liquid scintillation counting.

H. Chemotherapy Experiments with *in vivo* implants of Hepatoma 3924A Cells

Hepatoma 3924A was chosen for this study because lines of this neoplasm are available for both *in vivo* and *in vitro* passage. Experiments with cultured 3924A cells demonstrated that uptake of tubercidin by these cells was largely insensitive to NBMPR. A necessary preliminary to therapeutic trials with tubercidin-NBMPR-P combinations against ACI/N rats implanted with hepatoma 3924A was the demonstration that NBMPR-P protected rats of the inbred ACI/N line against tubercidin lethality, as in other experiments with Sprague-Dawley rats (Jakobs and Paterson, 1984) and with BDF_1 mice (Lynch *et al.*, 1981a,b). Following that demonstration, a chemotherapy experiment was undertaken in which rats bearing subcutaneous implants of 3924A cells were treated with the tubercidin-NBMPR-P combination by the i.p. route.

Dr. George Weber kindly provided ACI/N rats with subcutaneous implants of an *in vivo*-passaged line of hepatoma 3924A. The *in vivo*-passaged line of hepatoma 3924A was maintained by transplanting 3924A cells into new ACI/N hosts at intervals of about 14 days in the following way. Tumor tissue was obtained from tumors of the previous implant generation (usually about 2 cm in diameter and 13-16 days after implantation), using aseptic techniques. The solid tumor was freed of necrotic material and minced finely with scissors in 1 volume of Dulbecco's PBS. The mince was forced through a metal screen (100 mesh/linear inch) by means of a plastic pestle, and the final cell suspension was then forced by means of syringes through hypodermic needles of several sizes, with the final size being 26 gauge. For tumor maintenance and for chemotherapy experiments, 0.5-ml portions of such cell suspensions containing approximately 5 x 10^7 cells were implanted by subcutaneous injection in the dorsal-lumbar region of male ACI/N rats (100 - 200 g). Tumor dimensions were measured by vernier calipers (measurements in 2 directions at right angles to each other) twice weekly

and tumor masses were calculated using the following relationship:

$$\text{Tumor weight (mg)} = a \times b/2$$

where a and b are the tumor dimensions in mm (Looney *et al.*, 1983).

a. NBMPR-P Protection of ACI/N Rats Against Tubercidin

To determine if the tolerance (LD_{50}) of ACI/N rats toward tubercidin was increased by the administration of NBMPR-P, male ACI/N rats in groups of 4 were treated by i.p. injection with graded doses of tubercidin solution (0.3 - 2.5 mg/kg) in 0.154 M saline (NaCl). Four treatments were given, each 24 h apart. Rats in some groups received an i.p. injection of NBMPR-P solution (50 mg/kg) given 1 min before tubercidin was administered. Rats were weighed on treatment days and at 7-day intervals thereafter, and were checked daily and mortality recorded. Early signs of tubercidin toxicity were weight loss, diarrhea, ruffled fur and lethargy. Peritoneal effusion (clear, amber fluid) developed in 13% of the animals treated with tubercidin and in 8% of the animals given the tubercidin-NBMPR-P combination. Mortality was plotted against tubercidin dose to determine LD_{50} values.

b. Chemotherapy With the Tubercidin-NBMPR-P Combination

Chemotherapy with the tubercidin-NBMPR-P regimen was initiated 3 days after implantation, the time at which the tumors were first palpable. Rats in groups of 4 received the following agents by i.p. injection in a total of 4 treatments, each 24 h apart. The agents were dissolved in 0.154 M NaCl. Body weight and tumor size were monitored periodically.

(1) 0.154 M NaCl (control)

(2) tubercidin, (0.3 mg/kg)

(3) NBMPR-P and tubercidin (50 mg/kg and 1.6 mg/kg, respectively)

(4) NBMPR-P and tubercidin (75 mg/kg and 1.8 mg/kg, respectively)

IV. RESULTS

A. Failure of NBMPR to Protect Hepatoma 3924A Cells Against Tubercidin Toxicity

Experiments in which hepatoma 3924A cells were cultured in the presence and absence of tubercidin demonstrated the antiproliferative effects of this agent. In the experiment of Figure 3, cell proliferation was close to exponential for the first 48 h of culture in medium that lacked additives (control cultures). In the presence of 5 μM NBMPR, cell proliferation rates were similar to those of control cultures. In medium containing 1.5 μM tubercidin, cell numbers increased 1.3-fold during the first 24 hr of culture, but decreased thereafter, whether or not the culture medium contained 5 μM NBMPR. Thus, NBMPR, a potent inhibitor of nucleoside transport, failed to protect hepatoma 3924A cells against the cytotoxic effects of tubercidin. Earlier reports from this laboratory demonstrated that NBMPR at concentrations of 5 - 8 μM protected cells proliferating in culture (RPMI 6410 human lymphoblastoid cells (Paterson *et al.*, 1979b) and S49 mouse lymphoma cells (Cass *et al.*, 1981)) against extracellular lethal concentrations of tubercidin or other cytotoxic nucleosides during several days of culture. Since tubercidin is known to be a substrate for the NBMPR-sensitive, facilitated diffusion nucleoside transport system of L5178Y cells (Harley *et al.*, 1982), the inability of NBMPR to protect hepatoma 3924A cells against tubercidin suggested that the nucleoside transport system of the latter cells is of low sensitivity to NBMPR relative to the transporters of L5178Y and S49 cells.

The experiment of Figure 4 measured concentrations of tubercidin that reduced cell proliferation rates by 50% (IC_{50}) in the presence and absence of 5 μM NBMPR, in cultures of hepatoma 3924A cells. Cultures contained graded concentrations of tubercidin with or without 5 μM NBMPR, and cell concentrations were determined after 72 h of culture. Figure 4

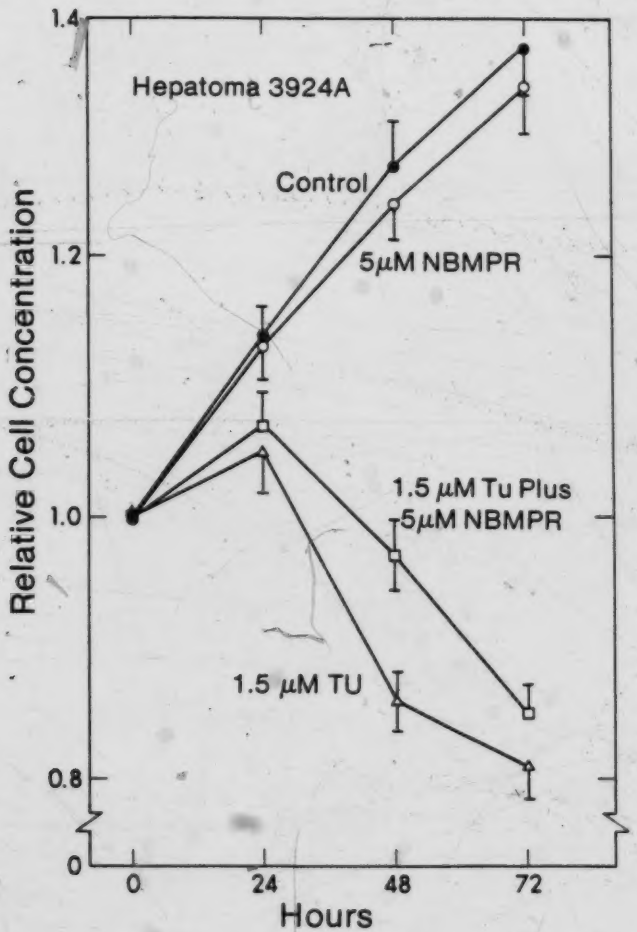

Figure 3. Failure of NBMPR to protect hepatoma 3924A cells against the cytotoxicity of tubercidin. Cells were cultured in medium without additives (●), or in medium containing 1.5 μM tubercidin (TU) with (□) and without (△) 5 μM NBMPR, or with only 5 μM NBMPR (○). The 2.5 ml cultures were prepared in triplicate and were sampled at the times indicated for determination of cell concentration.

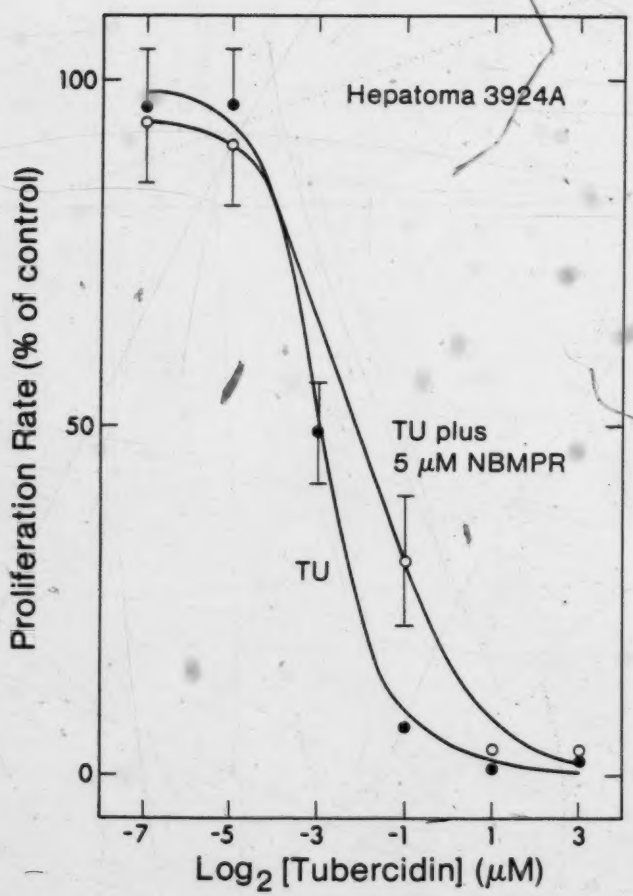

Figure 4. Effect of tubercidin on cell proliferation. Hepatoma 3924A cells were incubated in growth medium containing various concentrations of tubercidin (TU) in the presence (○) or absence (●) of 5 μM NBMPR. Proliferation rates (number of cell doublings during 72 h of culture) in the presence of these agents are expressed as percentages of proliferation rates in the absence of drug. Cultures were prepared in triplicate and means ± S.D. are plotted. The S.D. values that fell within the symbols are not shown.

indicates that cell proliferation rates (number of doublings that occurred during the 72-h interval of culture) were reduced by 50% at tubercidin concentrations of about 0.13 and 0.2 μM, respectively, in the presence and absence of 5 μM NBMPR.

The failure of NBMPR to protect hepatoma 3924A cells from the cytotoxic effects of tubercidin (Figure 3), and the similarity of the IC_{50} values for tubercidin inhibition of cell proliferation in the presence and absence of 5 μM NBMPR (Figure 4), indicate that the nulceoside transport system, presumed to be present in hepatoma 3924A cells, is of low sensitivity to NBMPR. An alternative possibility, a substantial non-mediated entry of tubercidin into 3924A cells during culture, is unlikely to account for the failure of NBMPR to protect. Previously reported evidence that non-mediated entry of nucleosides into cultured cells is a very small fraction (<1%) of mediated influx is discussed below (Sec. V. A).

B. Characterization of the Nucleoside Transport System in Hepatoma 3924A Cells

From the cytotoxic effects of tubercidin on hepatoma 3924A cells (Figure 3), it is evident that the cells are permeable to this agent. It has been demonstrated in earlier reports from this laboratory that cultured cells have low permeability to tubercidin if their nucleoside transport systems become inactivated by (i) mutation, as in the instance of AE_1 cells (as noted above) (Cass *et al.*, 1981)), or (ii) inhibitors of nucleoside transport (Paterson *et al.*, 1979b, 1983a,b). Thus, the presence of a nucleoside transport system in hepatoma 3924A cells may be reasonably inferred.

The existence of a nucleoside transport system in hepatoma 3924A cells was shown in kinetic studies of adenosine permeation that demonstrated saturability of transport rates. This is among the classical criteria for recognition of transporter-mediated entry of permeants into cells. The apparently low NBMPR sensitivity of this system was confirmed by direct measurements of adenosine transport in 3924A cells. The low sensitivity of the 3924A transport system to NBMPR invited comparison with other systems of low NBMPR sensitivity that have

been recognized in cultured cell lines of the Walker 256 carcinosarcoma (Belt and Noel, 1985) and the Novikoff hepatoma N1S1-67. Cells of the former line are devoid of the sites that bind NBMPR with high affinity (Plagemann and Wohlhueter, 1985), whereas, the existence of such sites on Novikoff UA cells has been shown (Gati *et al.*, 1986).

A surprising aspect of the characterization of the nucleoside transport system of 3924A cells was the presence of NBMPR binding sites in association with a nucleoside transport system of low NBMPR sensitivity.

a. Adenosine Transport in Hepatoma 3924A Cells

It is generally appreciated that nucleoside transport in animal cells is a rapid process and that rapid sampling methods are required in order to measure nucleoside fluxes (Paterson *et al.*, 1981a,b; Wohlhueter *et al.*,1978). In the present study, rates of the inward transport of nucleoside were measured as initial rates of nucleoside uptake determined from early time courses of cellular uptake, as indicated in an earlier section (see I. I.c). With cells in suspension, methods employed to rapidly end intervals of permeant uptake include removal of cells from the reaction system by centrifugation through an oil layer, or addition of transport inhibitors, such as NBMPR.

Neither of these approaches was possible in the present study because of the low NBMPR sensitivity of the 3924A transport system and because these cells require surface attachment. Although cells, for use in photolabelling experiments were cultured in 5-litre suspension flasks under vigorous agitation by the "Vibro-Mixer" system, accurate cell number could not be determined because the cells in suspension grew not as single cells, but as clumps of several cells that resisted trypsin treatment. In experiments that required accurate determination of cell number, such as binding and transport experiments, the cells were cultured as monolayers and intervals of permeant exposure were, of necessity, managed by changing medium. Intervals of permeant uptake were begun by rapidly flooding the

monolayers with medium containing ^{3}H-adenosine. To end uptake, the most of the medium was removed by suction about 2 sec before rapidly immmersing the culture dish into a large volume of ice-cold Dulbecco's PBS. Thus, intervals of uptake were ended by dilution and by cooling. With timing of these actions by metronome signals, the minimum, precise interval of permeant uptake with the monolayer cultures was 5 sec.

As indicated in Figure 5, the cellular uptake of [2,8-^{3}H]adenosine at graded concentrations was measured at intervals between 5 and 20 sec to construct time courses. Such time courses began with time-zero values obtained by (i) flooding monolayers with medium containing 10 mM non-isotopic adenosine for 10 sec, and (ii) exposing monolayers for the briefest interval possible to ice-cold medium containing ^{3}H-adenosine at the specified concentrations. Time-zero values for the adenosine content of the monolayers were approximately proportional to concentration, as might be expected if that parameter was determined by adsorption and other physico-chemical properties of the system. Time courses of adenosine uptake were appreciably curved and initial rates, estimated graphically from the time courses (see Materials and Methods), are plotted against adenosine concentration in Figure 6. These data demonstrate rate saturability and, therefore, indicate mediated permeation of adenosine. Analysis of the rate-concentration data by a Lineweaver-Burk (1934) plot (inset) yielded the following kinetic constants for adenosine transport: Km, 15 $\pm$ 3.2 μM; Vmax, 6.4 $\pm$ 0.7 pmoles/10^6 cells/sec. The Km value for adenosine transport obtained in this experiment agrees with reported values for adenosine influx in other cell types (Harley *et al.*, 1982). Thus, this experiment demonstrated the presence of a saturable, nucleoside transport system in hepatoma 3924A cells.

b. Inhibition of Adenosine Transport by NBMPR in Hepatoma 3924A Cells

As noted above, the inability of NBMPR to protect 3924A cells proliferating in culture against cytotoxic effects of tubercidin (Figure 3) implied that the nucleoside transport

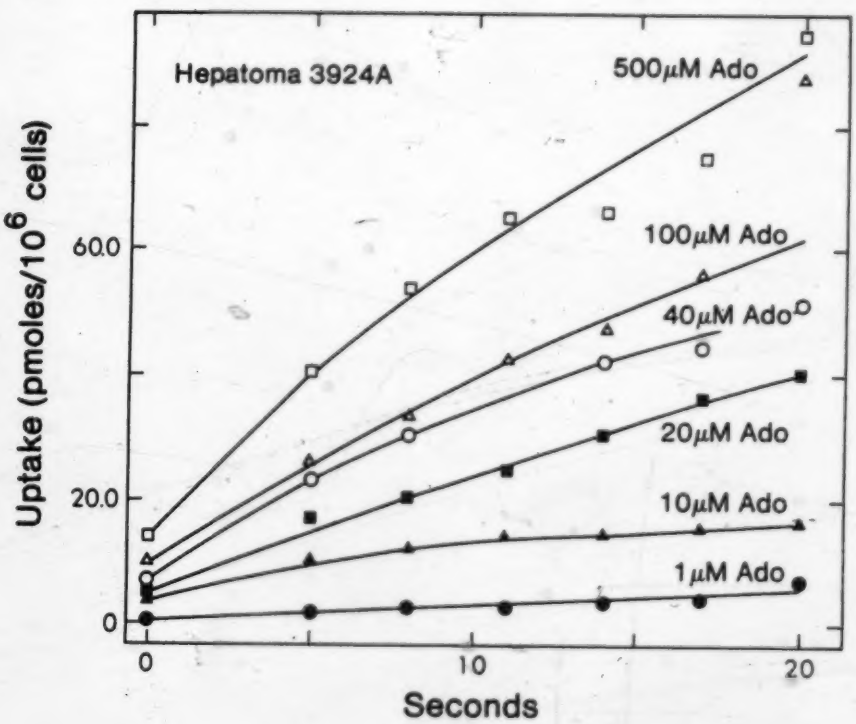

Figure 5. Time courses of adenosine uptake by replicate monolayer cultures of hepatoma 3924A cells. Intervals of adenosine uptake were started by addition of transport medium containing [2,8-^{3}H]adenosine (Ado), as described in the Materials and Methods; final concentrations are indicated. Intervals of uptake by hepatoma 3924A cells were ended by immersing culture dishes into ice-cold Dulbecco's PBS. Each point is the mean of triplicate assays.

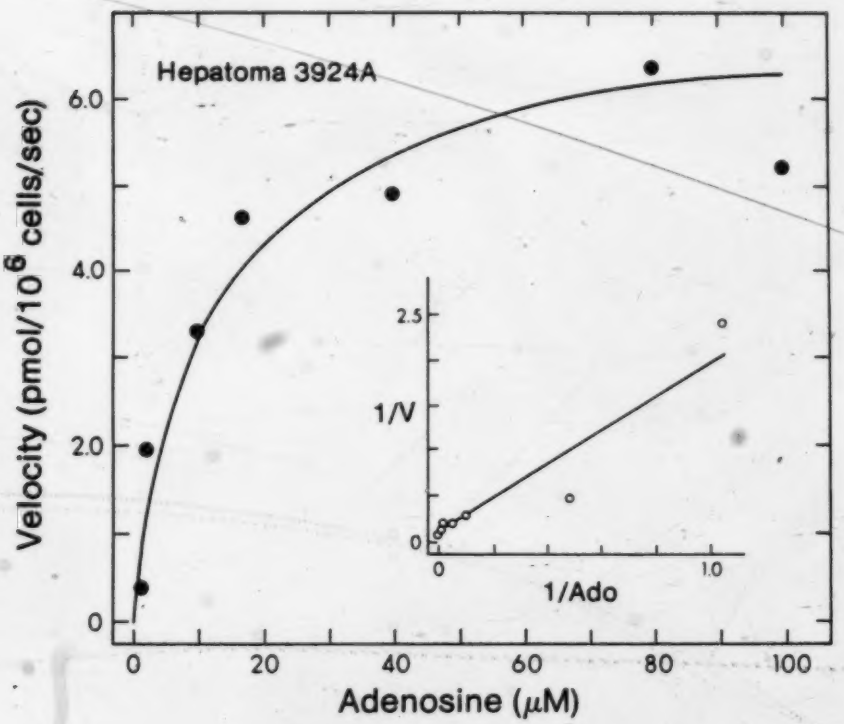

Figure 6. Transport of adenosine in hepatoma 3924A cells. Rates of [2,8-^{3}H]adenosine uptake by monolayer hepatoma 3924A cultures determined from the data in Figure 5 are plotted against adenosine concentrations. Analysis of these data by plotting reciprocals (inset) yielded Km and Vmax values of 15 μM and 6.4 pmoles/10^6 cells/sec, respectively.

mechanism of these cells is of low sensitivity to NBMPR. Earlier reports from this laboratory have noted the similar affinities (Km values) of tubercidin and adenosine for the nucleoside transport systems of several cell types (Harley *et al.*, 1982). Having shown the existence in hepatoma 3924A cells of a system that mediates the entry of adenosine, we then determined the sensitivity of this system to NBMPR, dilazep and dipyridamole, compounds known to inhibit the permeation of adenosine in other cell types.

In the experiment of Figure 7, replicate monolayer cultures of 3924A cells were equilibrated with graded concentrations of the inhibitors for 10 min prior to assays of cellular adenosine uptake rates in which cells were exposed to 20 μM [2,8-^{3}H]adenosine for 5 sec. Uptake rates measured with this assay approximated adenosine transport rates. Assay intervals were started by addition of transport medium containing adenosine and inhibitors at the concentrations indicated in Figure 7 and ended by immersion of culture dishes in cold Dulbecco's PBS. Adenosine permeation in the 3924A cells was relatively insensitive to inhibiton by NBMPR in that the IC_{50} value for that agent (Figure 7) was about 1 μM, whereas the corresponding value for S49 cells (data from Gati *et al.*, 1986) was about 5 nM. Results of similar experiments with dipyridamole and dilazep as inhibitors are also shown in Figure 7. The latter compounds are structurally unrelated to NBMPR, but are potent inhibitors of nucleoside transport in S49 cells and mouse leukemia L1210 cells (Belt, 1983). The concentrations of dilazep and dipyridamole that inhibited adenosine transport by 50% were about 3 μM and 1 μM, respectively. These experiments demonstrated important differences in NBMPR sensitivity between the nucleoside transport systems of hepatoma 3924A and S49 cells. Data yielding steep inhibitor concentration-effect plots, such as that obtained with S49 cells (Figure 7), often yield Hill coefficients near unity, suggesting simple inhibitor-site interactions without cooperative interactions between sites. The much shallower inhibitor concentration-effect plots obtained with 3924A cells, which extend over several logs of inhibitor concentration, may indicate Hill coefficients lower than unity, suggesting, perhaps, inhibitor binding to several transporter-associated sites.

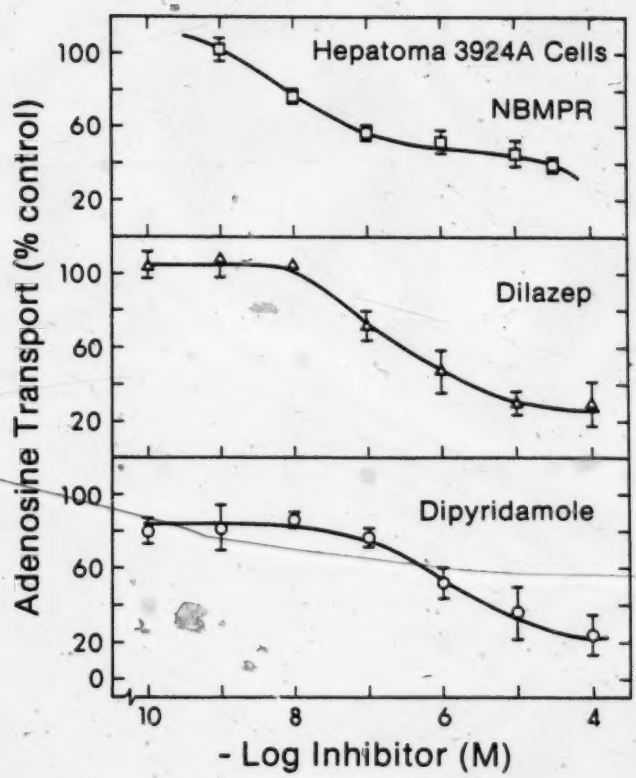

Figure 7. Inhibition of adenosine transport in hepatoma 3924A cells. Replicate monolayers were incubated at 22° in growth medium containing the indicated concentration of either NBMPR (□), dilazep (△) or dipyridamole (○) for 20 min and then assayed for rates of cellular uptake of [2,8-^{3}H]adenosine (20 μM) during uptake intervals of 5 sec, as described in Materials and Methods. Rates of adenosine uptake measured in this assay approximate transport rates. Means ± S.D. are plotted; in one instance, the symbol covers the error bars.

C. NBMPR Binding to Hepatoma 3924A Cells

Many types of animal cells possess NBMPR binding sites that are located in the plasma membrane and accessible at the external aspect of the membrane. Occupation of these sites by NBMPR correlated with inhibition of nucleoside transport in S49 mouse lymphoma cells (Cass *et al.*, 1981) and human erythrocytes (Cass *et al.*, 1974; Jarvis and Young, 1980). However, such sites were absent from cell types with transporters of low NBMPR sensitivity (Walker 256 (Paterson *et al.*, 1983a) and Novikoff N1S1-67(Wohlhueter *et al.*, 1978b)), supporting the idea that NBMPR binding sites identify functional nucleoside transporters (Young and Jarvis, 1983).

However, Novikoff UA cells, a subline of the Novikoff N1S1-67 cells line, were found to possess high affinity binding sites, yet NBMPR occupancy of these sites did not inhibit transporter function (Gati *et al.*, 1986). The present study also demonstrated the presence of NBMPR binding sites that do not appear to couple with the nucleoside transport system. Figure 8 indicates that [G-^{3}H]NBMPR became associated with 3924A cells during exposure to graded concentrations of the ligand by binding to specific sites and also by binding in a non-specific manner. Mass law analysis of these binding data (Figure 9) indicated that for hepatoma 3924A cells, Bmax, the maximum number of NBMPR-specific binding sites, and Kd, the dissociation constant for site-bound NBMPR, were 3 x 10^5 sites/cell and 1.8 nM, respectively. These values are similar to those previously reported for NBMPR binding to cultured cells of various lines (Lauzon and Paterson, 1977; Cass *et al.*, 1981; Koren *et al.*, 1983; Plagemann and Wohlhueter, 1985).

D. Relationship of NBMPR Binding Proteins to the Nucleoside Transport System

It is apparent in the data of Figures 7 to 9 that in 3924A cells, (i) high affinity binding sites would be mostly occupied by NBMPR at concentrations in excess of 10 nM, and (ii)

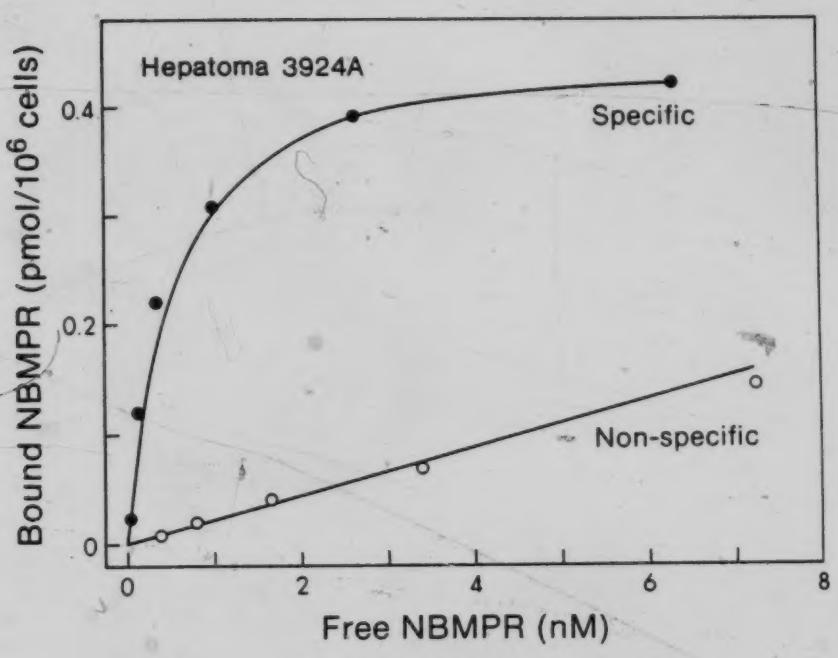

Figure 8. Presence of high affinity binding sites on hepatoma 3924A cells. Replicate monolayer cultures of hepatoma 3924A cells were exposed for 20 min at 22 ° to Dulbecco's PBS containing graded concentrations of ^{3}H-NBMPR with and without 5 μM non-isotopic NBMPR. The ^{3}H-contents of medium and cells were determined as described in Materials and Methods. Site-specific binding of ^{3}H-NBMPR (●) was determined as the difference between the cell content of ^{3}H-NBMPR in the absence of NBMPR (data not shown),or in the presence of (○) of 5 μM non-isotopic NBMPR. Means of triplicate assays are plotted.

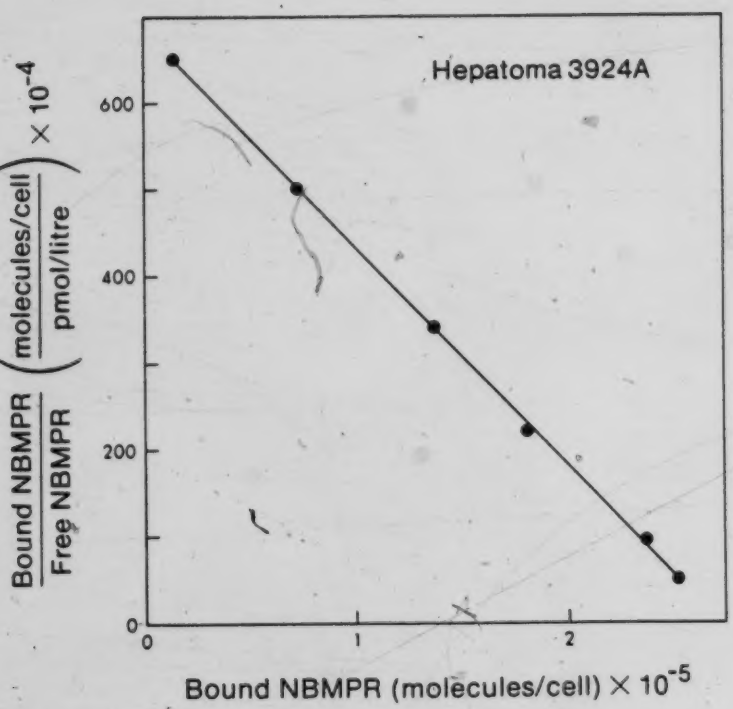

Figure 9. Scatchard plots of the data from Figure 8. Mass law analysis by the method of Scatchard (Edsall and Wyman, 1958) of data (from Figure 8) for the site-specific binding of NBMPR to 3924A cells under equilibrium conditions yielded the following binding constants for NBMPR: Kd, 1.2±0.006 nM; Bmax, (2.9±0.4)x10^5 sites/cell.

NBMPR inhibition of adenosine transport (IC_{50} about 1 μM, Figure 7) was not related to binding site occupancy by NBMPR. Thus, it appears that in these cells, the NBMPR-binding protein complex does not couple or interact with the adenosine transport system, as in the instance of the NBMPR-sensitive erythrocyte transporter (Cass *et al.*, 1974). It has been proposed that in the latter system, the NBMPR site may be an integral part of the nucleoside transporter (Young *et al.*, 1983). These considerations raise questions about the physical and functional relationships between the NBMPR binding protein and the nucleoside transport system in 3924A cells. This relationship was explored in the present study by determining whether the presence of adenosine and uridine, substrates for nucleoside transport systems, affected NBMPR binding at the high affinity sites. As well, the influence of dilazep, a potent inhibitor of nucleoside transport, on NBMPR binding to 3924A cells was examined.

a. Adenosine Inhibition of NBMPR Binding

The experiment of Figure 10 measured the effect of graded concentrations of adenosine on the site-specific binding of NBMPR to 3924A hepatoma cells. Replicate monolayer cultures were incubated for 20 min to allow equilibrium to be established between free and site-bound NBMPR. As Figure 10 shows, the presence of adenosine at concentrations about 3- and 30-fold higher than the adenosine transport Km interfered with the site-specific binding of NBMPR. The very limited data of Figure 10 on the concentration-dependence of the adenosine inhibitory effect were analyzed by means of the reciprocal plot shown in Figure 11. The results suggest that adenosine competed with NBMPR at the high affinity binding sites with a Ki of about 180 μM . Since the Km value for the interaction of adenosine with the nucleoside transport system was found to be 15 μM (Figure 6), that interaction would not likely be the basis of the adenosine inhibition of NBMPR binding. This result argues that the adenosine permeation sites and the NBMPR binding sites are separate entities in these cells.

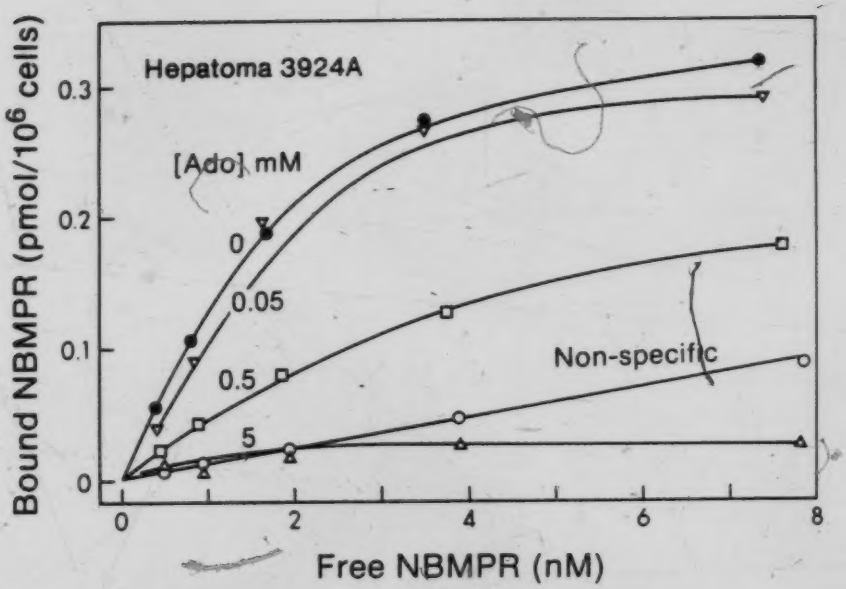

Figure 10. Adenosine inhibition of site-specific NBMPR binding to hepatoma 3924A cells. Replicate monolayer cultures of hepatoma 3924A cells were incubated for 20 min at 22° with Dulbecco's PBS containing graded concentrations of [G-^{3}H]NBMPR with adenosine at 0.050 mM (▽), 0.5 mM (□), 5 mM (△), without adenosine (●), or with 5 μM non-isotopic NBMPR (○, non-specific). The ^{3}H-contents of medium and cells were determined as described in Materials and Methods and site-specific binding was determined as in Figure 8. Means of triplicate determinations are plotted.

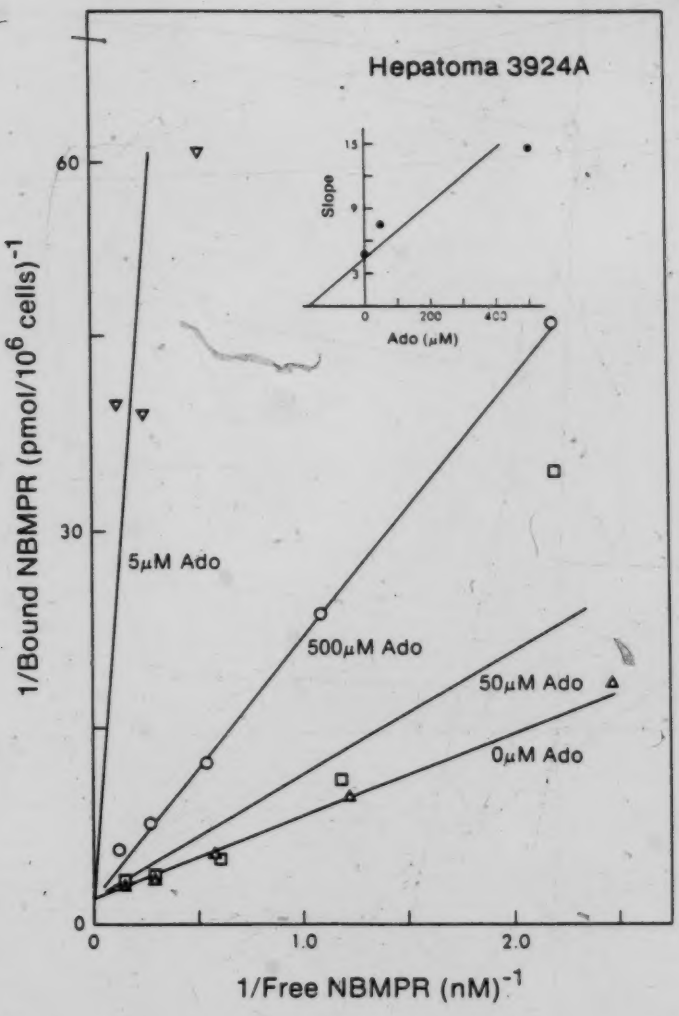

Figure 11. Adenosine inhibition of site-specific NBMPR binding in hepatoma 3924A cells. This figure represents a mass law analysis by the reciprocal plot method (Edsall and Wyman, 1958) of data from Figure 10 for the site-specific binding of ^{3}H-NBMPR in the absence of adenosine (△) and in the presence of 0.05 mM (□), 0.5 mM (○) and 5 mM (▽) adenosine. Site-specific binding is defined in Figure 8. The Kd value for site-bound NBMPR in the absence of adenosine was 1.8 nM. The Ki value for adenosine inhibition of NBMPR binding, determined by unweighted linear regression (inset), was 180 μM.

b. Uridine Inhibition of NBMPR Binding

The effect of uridine on NBMPR binding to hepatoma 3924A cells was measured in an experiment similar to that of Figures 10 and 11, the results of which are summarized in Figure 12. Site-specific binding of the ligand was inhibited in a concentration-dependent manner in the presence of uridine at concentrations in the 0.5 - 5 mM range. When the ligand binding-concentration data of Figure 12 were subjected to reciprocal plot analysis (Figure 13), linear plots that intersected at the ordinate were obtained, suggesting that uridine inhibited NBMPR binding competitively. It is widely appreciated that apparently competitive inhibitory effects may also arise from allosteric effects of inhibitors. Figure 13 includes an inset replot of apparent Kd values for NBMPR binding in the presence of various uridine concentrations. The replot indicates that uridine inhibited NBMPR binding with a Ki of about 0.5 mM, a value about 3- to 5-fold higher than the Km for the inward transport of uridine in cultured cells (Cass *et al.*, 1981; Belt and Noel, 1985).

These results show that both pyrimidine and purine nucleosides inhibit NBMPR binding and suggest that inhibition may be related to the relative "transportability" of the inhibiting nucleoside. Thus, the Ki value for uridine inhibition of NBMPR binding is 3 to 5-fold higher than that for adenosine, and Km values for uridine transport in several cell types are 3 to 5-fold higher than those for adenosine (Cass *et al.*, 1981; Belt and Noel, 1985; Paterson *et al.*, 1985). The significance of these results with respect to the physical relationship between the site-specific binding of NBMPR and the nucleoside transport site will be discussed below (see Part V).

c. Dilazep Inhibition of NBMPR Binding

Dilazep, dipyridamole and hexobendine have been recognized as potent inhibitors of nucleoside transport in various cell types. The experiment of Figure 14 demonstrates that

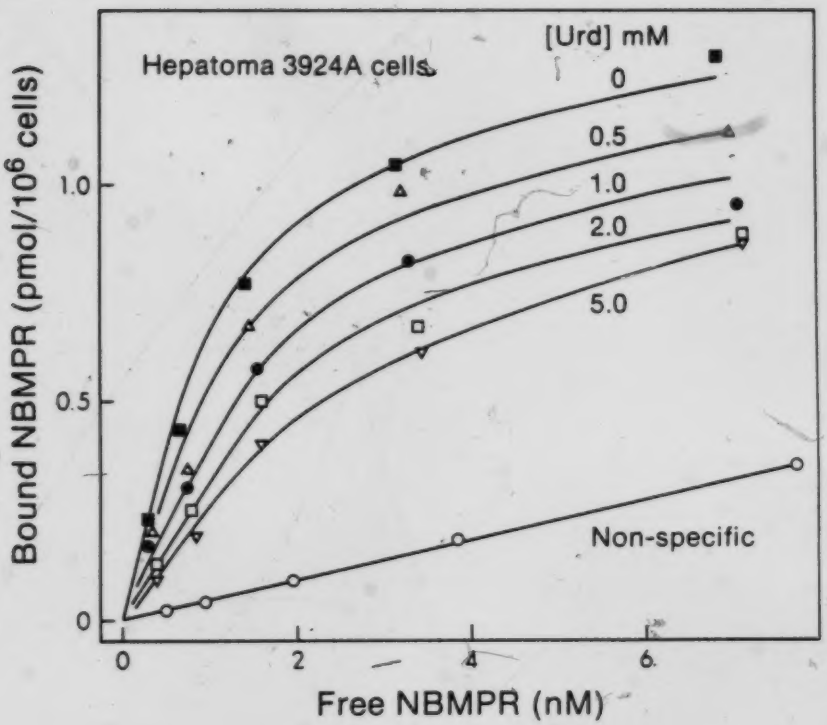

Figure 12. Uridine inhibition of site-specific NBMPR binding in hepatoma 3924A cells. Replicate monolayer cultures of hepatoma 3924A cells were incubated for 20 min with Dulbecco's PBS containing graded concentrations of [G-^{3}H]NBMPR and of uridine. The ^{3}H-content of cells and medium were determined as described in Materials and Methods and site-specific binding was determined as in Figure 8. Means of triplicate assays are plotted.

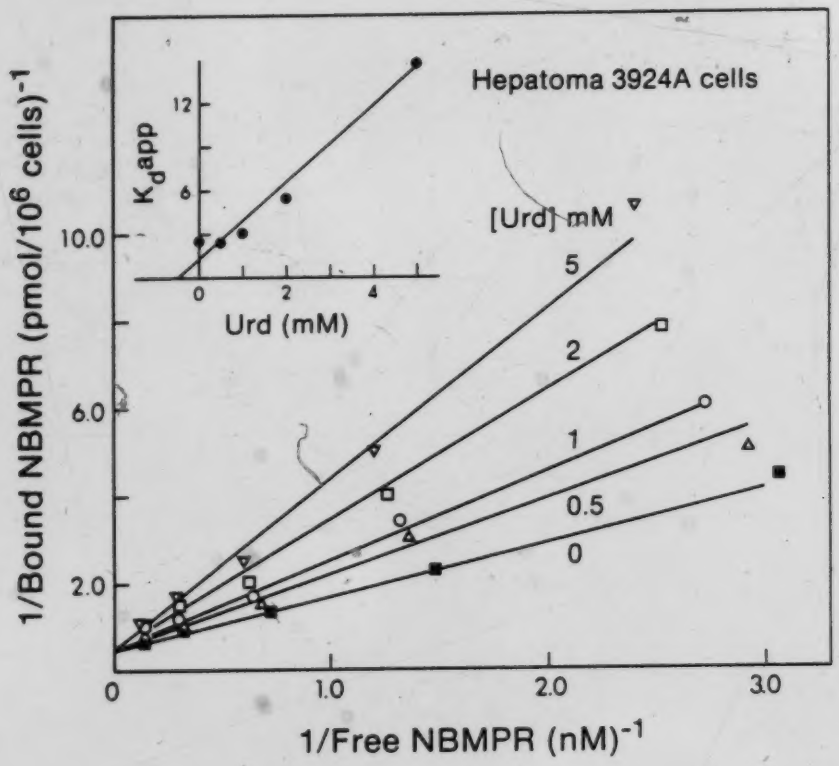

Figure 13. Reciprocal plots for uridine inhibition of NBMPR binding to hepatoma 3924A cells. In this figure, data from Figure 12 for the site-specific binding of ^{3}H-NBMPR (defined in Figure 8) in the absence of uridine (■) and in the presence of the specified concentrations of uridine are subjected to mass law analysis by the reciprocal plot method, as in Figure 11. The Kd value for site-bound NBMPR in the absence of uridine was 2.1 nM. The replot (inset) of apparent Kd values yielded a Ki value of 2.5 mM for the inhibition of NBMPR binding by uridine.

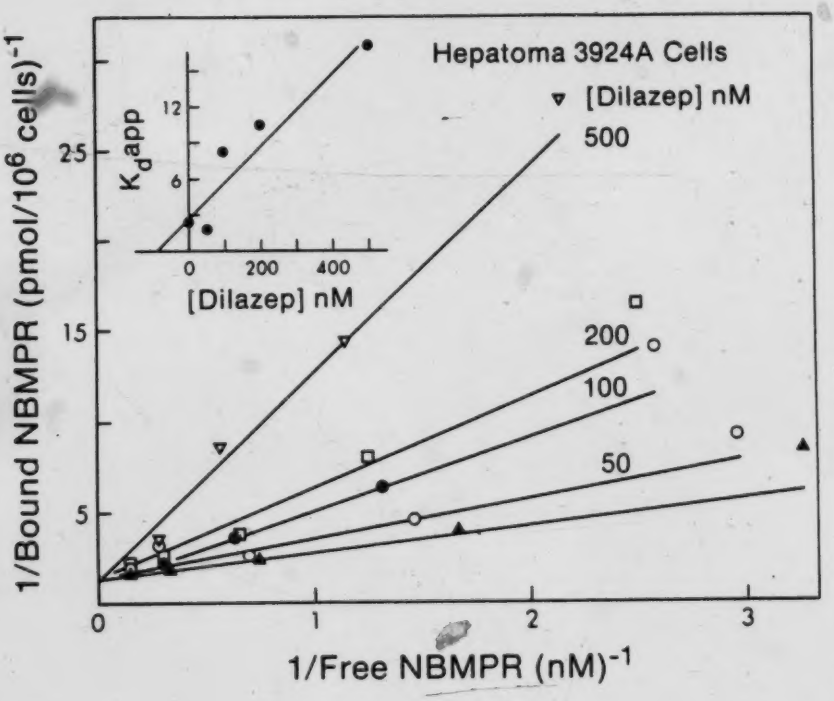

Figure 14. Dilazep inhibition of site-specific binding of NBMPR to hepatoma 3924A cells. Replicate monolayer cultures of hepatoma 3924A cells were incubated for 20 min at 22° in the presence of graded concentrations of [G-^{3}H]NBMPR, with 5 μM non-isotopic NBMPR (data not shown), in the absence of dilazep (▲), with dilazep at the indicated concentrations. Incubations were conducted in triplicate. Data for site-specific binding of NBMPR at particular dilazep concentrations were subjected to mass law analysis (this figure) by the reciprocal plot method, as in the experiment of Figures 10 and 11. The replot (inset) of the apparent Kd values (from the reciprocal plots) indicates that the Ki value for the inhibition of NBMPR binding by dilazep was 70 nM.

dilazep is a potent inhibitor of NBMPR binding in hepatoma 3924A cells. NBMPR binding to heptoma 3924A cells was inhibited in the presence of dilazep in a concentration-dependent manner. Reciprocal plot analysis of binding data (Figure 14) indicated that (i) dilazep inhibited NBMPR binding in an apparently competitive fashion, and (ii) the Ki for the inhibition of NBMPR binding by dilazep was 70 nM (Figure 14, inset). An earlier report from this laboratory indicated that dilazep was a potent and apparently competitive inhibitor (Ki, 15 nM) of NBMPR binding to N1L 8 hamster fibroblasts (Koren *et al.*, 1983).

E. The Nucleoside Transport System of Low NBMPR Sensitivity in Reuber Hepatoma H-35 Cells

Nucleoside transport systems of low sensitivity to NBMPR have been reported in cultured cell lines of the following rat neoplasms: Morris hepatoma 3924A, Novikoff hepatoma N1S1-67 (UA subline), and Walker 256 carcinosarcoma. Sites that bind NBMPR with high affinity have been detected in cells of two of these lines, hepatoma 3924A and Novikoff UA. It will be of some importance to determine whether low NBMPR sensitivity and the presence of "uncoupled" NBMPR binding sites are species-related, or hepatoma-related characteristics. As a step in this direction, cells of a cultured line of the Reuber H-35 rat hepatoma were examined for these characteristics.

a. Inhibition of Nucleosoide Transport in H-35 Cells

As in the experiments with hepatoma 3924A cells just described, the sensitivity of nucleoside transport in H-35 cells to NBMPR, dilazep and dipyridamole was evaluated. The rate of adenosine uptake by monolayers of H-35 cells from medium containing 20 μM ^{3}H-adenosine was constant between 0 and 8 sec of exposure (data not shown) and, accordingly, the cellular uptake of adenosine during the first 5 sec of exposure under these conditions was

employed as an assay of adenosine transport rates. Adenosine transport rates in H-35 cells after 20 min of incubation in the presence of graded concentrations of NBMPR are reported in Figure 15. These data indicate that (i) adenosine transport was relatively insensitive to NBMPR when the ligand was present at nanomolar concentrations, and (ii) the IC_{50} value for NBMPR inhibition of adenosine transport was about 5 μM. The effects of prior incubation with dilazep and dipyridamole on nucleoside transport are also shown in Figure 15. The inhibitory effects of these compounds were evaluated in a manner similar to that of NBMPR. The IC_{50} values for the inhibition of adenosine transport by dilazep and dipyridamole were 10 and 0.9 μM, respectively. Thus, it appears that the inhibitor sensitivity of the adenosine transport system in H-35 cells is similar to that of hepatoma 3924A. The shallow NBMPR concentration-effect curves may indicate interaction of NBMPR with several sites associated with the nucleoside transport system(s) of H-35 cells.

b. Site-specific Binding of NBMPR to H-35 Cells

As part of our characterization of nucleoside transport of in H-35 cells, site-specific binding of NBMPR was evaluated by incubating monolayers with graded [G-^{3}H]NBMPR concentrations in the presence and absence of excess non-isotopic NBMPR. The experiment of Figure 17 -binding relationships in H-35 cells under equilibrium conditions. The binding constants, Kd and Bmax, obtained by Scatchard analysis (Figure 17) were 2.6 nM and 3 x 10^5 sites/cell, respectively. The linearity of the plot indicates the presence of a single class of sites that bind NBMPR with high affinity. The binding constants are similar to those of hepatoma 3924A cells. By these criteria, H-35 cells appear to possess a nucleoside transport mechanism that is similar to those of heptoma 3924A and Novikoff UA cells.

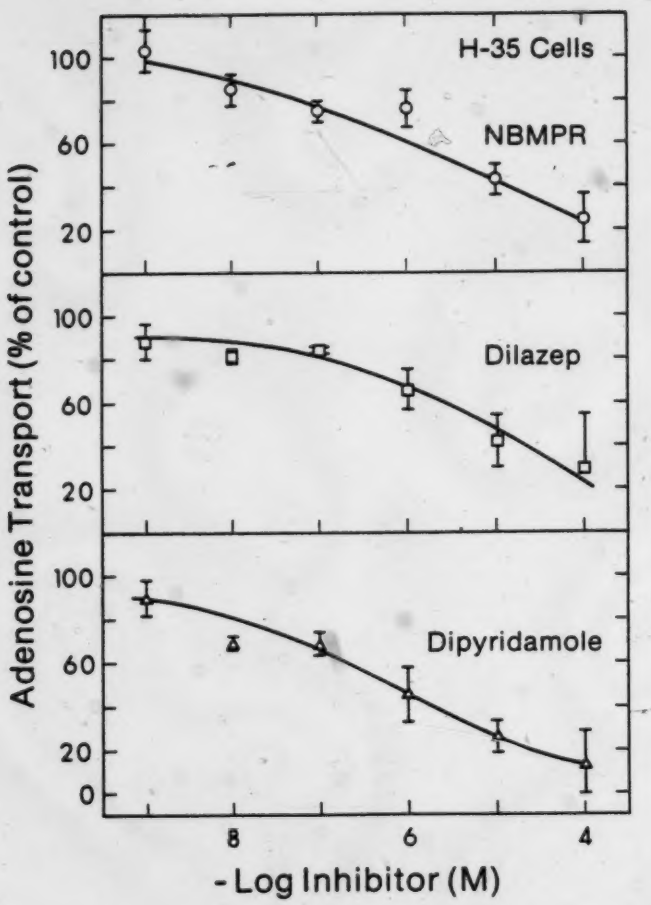

Figure 15. Inhibition of adenosine transport in H-35 cells. Cells were incubated with the indicated concentration of either NBMPR ( ), dilazep ( ) or dipyridamole ( ) for 20 min prior to assays for adenosine transport activity as described in Figure 5 and in Materials and Methods. Means ± S.D. are plotted.

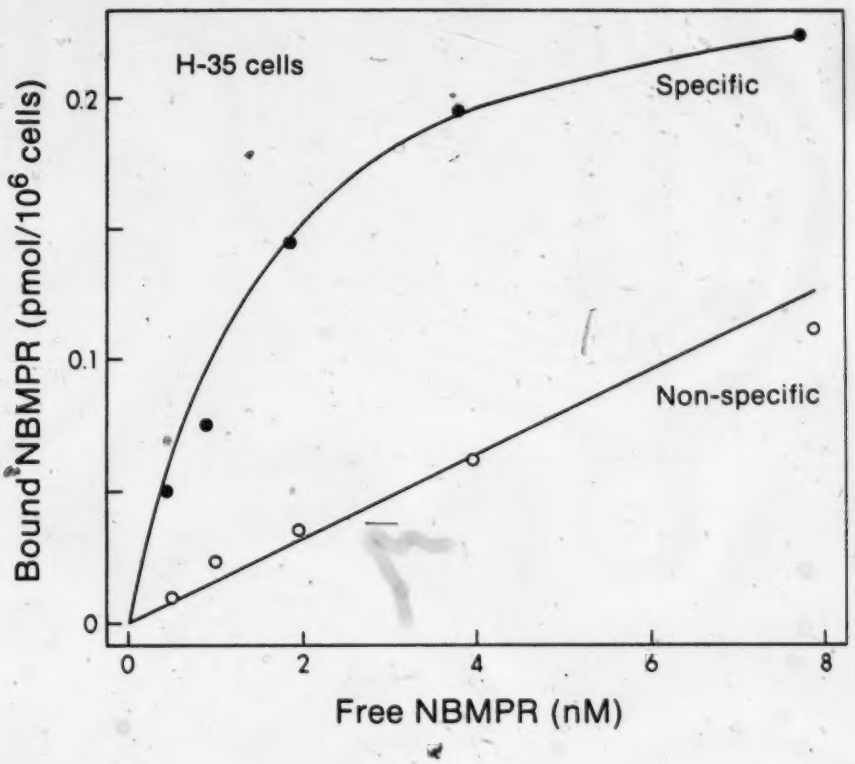

Figure 16. Presence of binding sites with high affinity for NBMPR on H-35 cells. Replicate monolayer cultures were exposed for 20 min at 22 ° in Dulbecco's PBS containing the indicated concentrations of [G-^{3}H]NBMPR without (data not shown) or with (○, non-specific) 5 μM non-isotopic NBMPR. Site-specific binding of NBMPR (●) is the difference between the NBMPR content of cells in the absence and presence of 5 μM non-isotopic NBMPR. Means of triplicate assays are plotted.

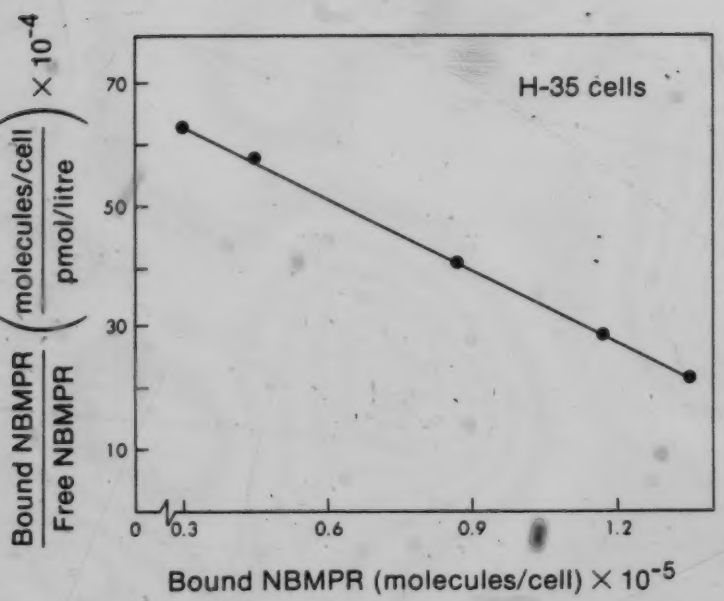

Figure 17. Mass law analysis by the method of Scatchard of Figure 16 data on site-specific binding of NBMPR to H-35 cells. This analysis yielded these binding constants: Kd, 2.62±0.56 nM; Bmax, (2.0±0.3)x10⁵ sites/cell.

c. Adenosine Inhibition of NBMPR Binding

Figure 18 shows the inhibitory effect of graded concentrations of adenosine on NBMPR binding to H-35 monolayers. Reciprocal plots of site-specific NBMPR binding *versus* NBMPR concentration (Figure 19) intersected at the ordinate indicating that the maximum number of NBMPR binding sites was the same in the presence and absence of adenosine, suggesting competition between adenosine and the ligand at the latter's binding site.

F. Photoaffinity Labelling of the Nucleoside Transporter in Cells of Rat Hepatomas 3924A and H-35

Covalent labelling of the membrane sites that bind NBMPR occurs when site-bound ^{3}H-NBMPR is activated by exposure to UV light. The binding site polypeptides so labelled may be identified by SDS gel electrophoresis. The NBMPR-binding proteins of the hepatoma cell lines employed in this study were characterized by this means. Crude membrane preparations rather than intact cells were used for this purpose.

Following 15 min of equilibration with ^{3}H-NBMPR, membrane-containing preparations from the cultured hepatoma cells were exposed to UV light, washed, dissolved in SDS-containing buffer and then subjected to polyacrylamide gel electrophoresis. Covalently bound ^{3}H-NBMPR migrated as a band in electropherograms of membrane preparations that had been exposed to [G-^{3}H]NBMPR alone, but such bands were absent from samples photolabelled in the presence of 10 μM non-isotopic NBTGR (Figure 20). Photolabelled membrane preparations from rat hepatoma 3924A cells contained an NBMPR-binding protein that migrated on electropherograms with a Mr of 80,000 - 100,000 (Figure 20), whereas, electropherograms of preparations from H-35 cells showed a labelled band that migrated with a Mr value between 45,000 and 64,000 (Figure 21). The NBMPR-binding protein of human erythrocyte membranes identified by this procedure has a Mr of 45,000-66,000 (Wu *et al.*,

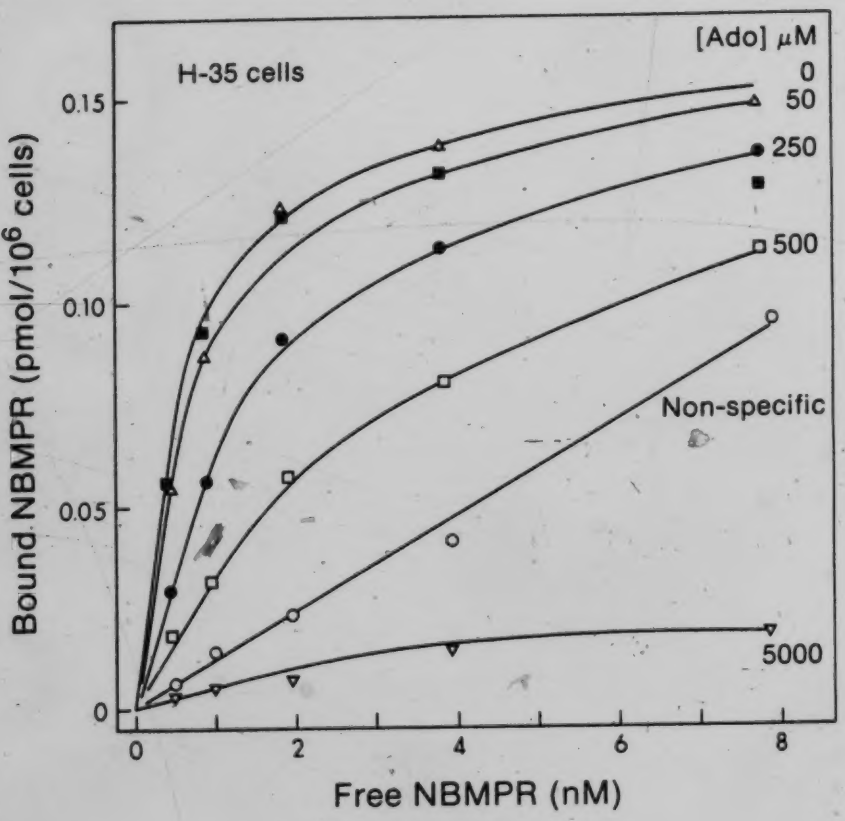

Figure 18. Adenosine inhibition of site-specific NBMPR binding to H-35 cells. Replicate monolayer culture of H-35 cells were incubated for 20 min with Dulbecco's PBS containing graded concentrations of [G-^{3}H]NBMPR with 5 μM non-isotopic NBMPR (○, non-specific), without adenosine (■), or with adenosine at the concentrations specified (filled symbols). The ^{3}H-contents of cells and medium were determined as specified in Materials and Methods, and data for site-specific binding NBMPR are shown, except as noted. Means of triplicate assays are plotted.

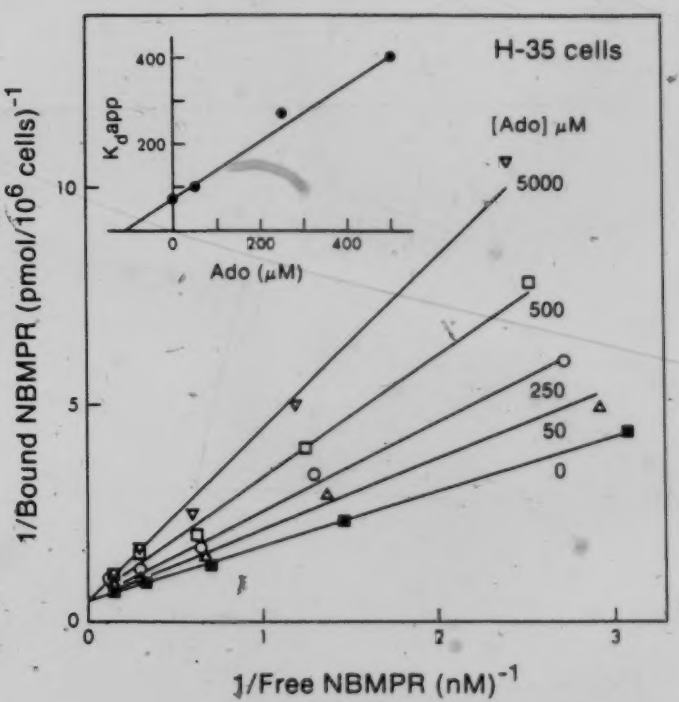

Figure 19. Adenosine inhibition of NBMPR binding in H-35 cells. In this figure, data from Figure 18 for the site-specific binding of ^{3}H-NBMPR in the absence of adenosine (■) and in the presence of the specified concentrations of adenosine are subjected to mass law analysis by the reciprocal plot method, as in Figure 11. The Kd value for NBMPR binding in the absence of adenosine was 0.71 nM. The replot (inset) of apparent Kd values for NBMPR binding in the presence of adenosine indicates that the Ki for adenosine inhibition of binding was 112 μM.

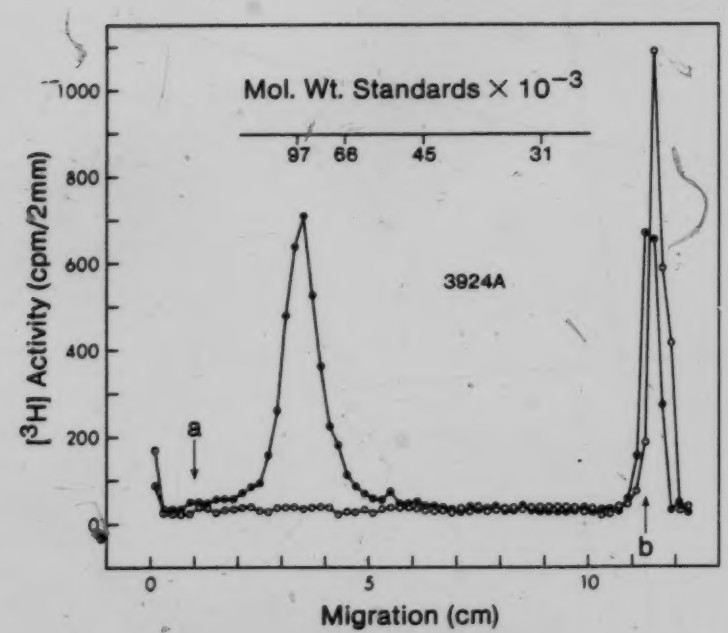

Figure 20. Photoaffinity labelling of NBMPR binding sites in membrane preparations from hepatoma 3924A cells. Samples of a crude plasma membrane preparation from hepatoma 3924A cells were equilibrated with 50 nM [G-³H]NBMPR in the presence (○) and absence (●) of 10 μM NBTGR and exposed to UV light for 1 min un the presence of 50 mM dithiothreitol. After washing to remove unreacted [G-³H]NBMPR, samples were dissolved in gel sample buffer and subjected to SDS-polyacrylamide gel electrophoresis as described in Materials and Methods. ³H-Profiles for the samples and the positions of the molecular weight standards are from the same gel (a, stacking gel/running gel interface; b, position of the tracking dye).

1983).

G. *In vivo* Experiments With The Tubercidin-NBMPR-P Combination

Figure 22 illustrates the protection afforded to ACI/N rats by treatment with NBMPR-P against doses of tubercidin that would have been lethal in the absence of the protectant. The LD_{50} value for tubercidin administered with protecting doses of NBMPR was 1.9 mg/kg, while that for tubercidin alone was 0.9 mg/kg. This experiment showed that ACI/N rats tolerated the tubercidin-NBMPR-P regimen; that regimen was subsequently used in the chemotherapy experiment described below. The i.p. administration of the protectant, NBMPR-P, 1 min before tubercidin, evidently modulated the tissue distribution of tubercidin with the result that vital, dose-limiting tissues were protected from the toxicity of tubercidin.

When ACI/N rats bearing subcutaneous implants of hepatoma 3924A were treated by the i.p. route with either the tubercidin or tubercidin-NBMPR-P regimens used in the experiment of Figure 22, there was no significant reduction in tumor size in animals treated with either regimen, relative to those treated with saline. It is likely that therapeutic concentrations of tubercidin in the vicinity of the tumor were not achieved because of metabolism of the drug by the liver.

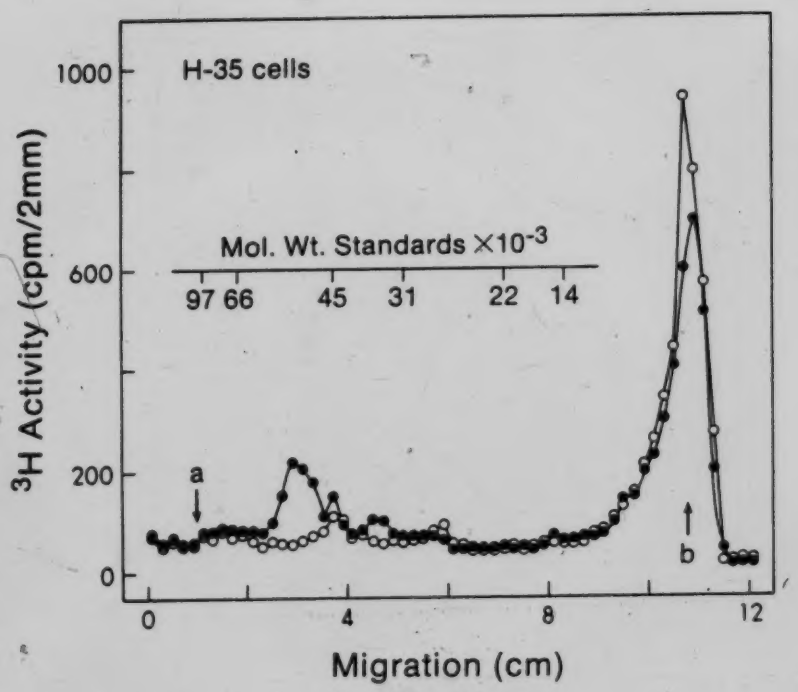

Figure 21. Photoaffinity labelling of NBMPR binding sites in membrane preparations from H-35 cells. Samples of a crude plasma membrane preparation from H-35 cells were equilibrated with 50 nM [G-^{3}H]NBMPR in the presence (○) and absence (●) of 10 μM NBTGR and exposed to UV light for 1 min un the presence of 50 mM dithiothreitol. After washing to remove unreacted [G-^{3}H]NBMPR, samples were dissolved in gel sample buffer were subjected to SDS-polyacrylamide gel electrophoresis as described in Materials and Methods. ^{3}H-Profiles for the samples and the positions of the molecular weight standards are from the same gel (a, stacking gel/running gel interface; b, position of the tracking dye).

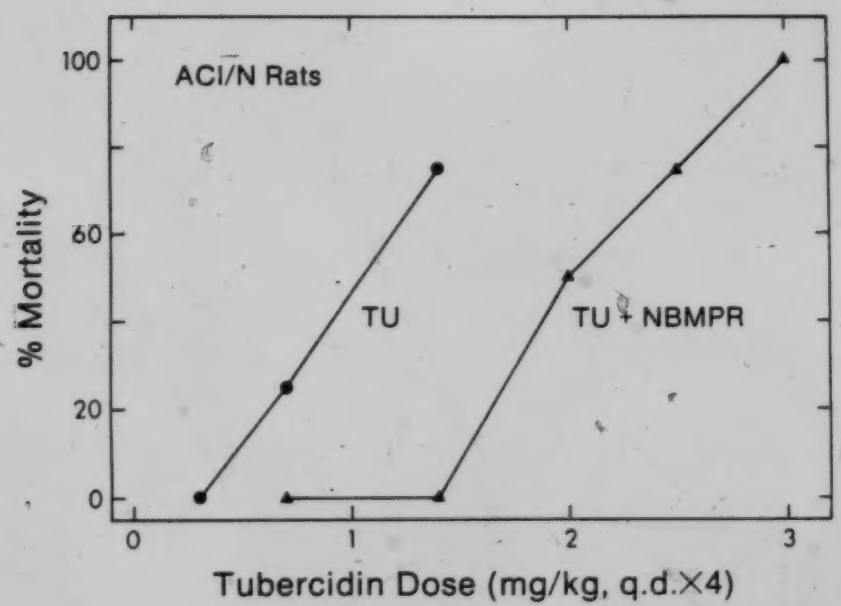

Figure 22. Protection of ACI/N rats by NBMPR-P against high, potentially lethal doses of tubercidin. Male ACI/N rats in groups of 4 were treated by the i.p. route with tubercidin administered alone (●) at the doses indicated, or with NBMPR-P (▲), (20 mg/kg) given 1 min before the indicated doses of tubercidin. Deaths were recorded for the next 30 days.

V. DISCUSSION

Nucleoside transport systems of low NBMPR sensitivity (IC_{50}, $> 1\ \mu M$) were recognized first in cultured cell lines derived from two rat neoplasms, the N1S1-67 Novikoff hepatoma (Wohlhueter *et al.*, 1978a) and the Walker 256 carcinosarcoma (Paterson *et al.*, 1983a; Belt and Noel, 1985; Jakobs and Paterson, 1984; Gati *et al.*, 1986). In the Walker 256 cell line, the insensitivity of the nucleoside transport system to NBMPR was consistent with the absence of sites with high affinity for NBMPR, that is, sites of the type found in association with transporters of high sensitivity to NBMPR[4]. However, the N1S1-67 line of the Novikoff hepatoma has proven to be heterogenous in that (i) cells from the Novikoff UA line (Gati *et al.*, 1986), and (ii) several clones from the latter (J.A. Belt, personal communication), have the same low NBMPR sensitivity as the N1S1-67 parent line of cells, yet possess sites that bind NBMPR with high affinity. The presence of NBMPR binding sites in cells with nucleoside transport systems of low NBMPR sensitivity suggests that in these cell types, the NBMPR-binding protein complex does not interact with the nucleoside transport mechanism in ways that result in transporter inhibition.

Zhen *et al.* (1983) demonstrated with cultured hepatoma 3924A cells that (i) the provision of exogenous nucleosides overcame the antiproliferative effects of acivicin, and (ii) dipyridamole, but not NBMPR, blocked nucleoside utilization by hepatoma 3924A cells. These results suggest that hepatoma 3924A cells possess a nucleoside transport system of low NBMPR sensitivity. The present study demonstrates that this inference was correct and, further, that 3924A cells possess high affinity NBMPR binding sites that, when occupied by NBMPR, do not block nucleoside transport.

A. Adenosine Transport in Hepatoma 3924A Cells

Nucleoside transport in animal cells is a rapid process. As a consequence, it has been necessary to develop rapid sampling methods to define the kinetics of nucleoside permeation during the first few seconds of cell exposure to permeant. The methods developed include (i) "instantaneous" inhibition of nucleoside transport by inhibitors, such as NBMPR or dilazep, in cells with transport systems that are sensitive to these compounds, and (ii) rapid centrifugation of cells through an oil layer to isolate them from permeant-containing medium using fast-starting microcentrifuges such as the Eppendorf Model 5412. With such methods, time courses that define the first 5 - 10 sec of cellular uptake of nucleoside permeants may be achieved. The selection of one method over the other depends largely on nucleoside transporter characteristics of the cells used. Of course, the use of NBMPR, or of other transport inhibitors such as dilazep, as chemical stoppers of nucleoside uptake, requires sensitivity of the cellular transport system(s) to the inhibitors. These methods are employed with cells in suspension.

Because hepatoma 3924A cells were cultured as monolayers in this study,[9] and because preliminary experiments indicated that the nucleoside transport system in these cells is relatively insensitive to NBMPR, dipyridamole and dilazep, neither of the above approaches to the measurement of nucleoside transport rates was without difficulties. A method for following the kinetics of nucleoside uptake was developed in which replicate monolayers of 3924A cells were exposed to graded concentrations of ^{3}H-permeant for brief time intervals (Figure 9). Intervals of permeant uptake of 5 sec or more were ended by rapid immersion of culture dishes into ice-cold Dulbecco's PBS to end cellular uptake of isotopic permeant by cooling and by dilution. An important aspect of this procedure was measurement of the cellular content of ^{3}H-permeant after an uptake interval of "zero seconds". Our approach to the measurement of "time-zero uptake" of ^{3}H-adenosine by cells involved exposure of cells to high concentrations

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[9] Hepatoma 3924A cells may be propagated in "Vibro-Mixer" suspension cultures, as noted in Materials and Methods; however, in such cultures, cells were found mainly as clusters of several adherant cells, making cell enumeration difficult. The use of monolayer cultures circumvented this problem.

(10 mM) of non-isotopic adenosine immediately prior to the briefest possible exposure of the cells to the labelled permeant.

Time courses of adenosine uptake by the monolayer cells obtained in this way over a range of adenosine concentrations (Figure 5) were approximately linear between 0 and 8 sec and, therefore, defined initial rates of adenosine uptake. Initial rates of permeant uptake, by definition, are a measure of transport, as noted above. Beyond 8 sec, the time courses of adenosine uptake were appreciably curved. Time-zero values of adenosine uptake were approximately proportional to the concentration of the permeant used, suggesting that adsorption, simple diffusion and non-specific binding of the permeant in the experimental system might account for such binding.

Measured in this way, adenosine uptake rates in 3924A cells were saturable, as seen in Figure 10, demonstrating that adenosine entry was transporter-mediated in these cells. The Km value of 15 μM for adenosine transport in hepatoma 3924A cells obtained in this study is similar to values for other cell types obtained by quenched-flow or "inhibitor-stop" methods (Paterson *et al.*, 1984, 1985; Paterson and Cass, 1986), indicating that this transport system has a high affinity for adenosine relative to reported Km values for inward transport of nucleosides in other cell types (Plagemann and Wohlhueter, 1980).

It should be noted that in the above-described procedure for measurement of adenosine transport rates, corrections were not made for permeant entry by non-mediated processes such as simple diffusion. In earlier work from this laboratory, rates of non-mediated entry of nucleosides into cultured cells were shown to be very small fractions of mediated entry rates. For example, rates of uridine transport in S49 cells at 22° were saturable (Km, 103 μM; Vmax, 3.9 pmol/μl pellet water/sec), but rates of uridine uptake in nucleoside transport-deficient AE_1 cells were less than 1% of those in S49 cells and required uptake intervals of 0.5-4 min for definitive rate measurements (Cass *et al.*, 1981). The same study showed that proliferation rates of AE_1 cells were 700- and 280-fold less sensitive than S49 cells to the cytotoxic nucleosides, showdomycin and tubercidin, and that in the presence of 5-8 μM

NBMPR, S49 cells were 520- and 730-fold less sensitive to these agents than in the absence of NBMPR. These results indicate that rates of non-mediated entry of cytotoxic nucleosides were less than 1% of rates of mediated entry. Further to this point, other studies from this laboratory have shown that proliferation rates of RPMI 6410 cells in culture medium containing 5μM NBMPR plus otherwise inhibitory concentrations of tubercidin, toyocamycin, or nebularine were largely unimpaired during 3-day intervals of culture (Paterson *et al.*, 1976b). Wohlhueter and Plagemann (1980) have also pointed out that nucleoside entry into cultured cells by simple diffusion is a very minor process.

The aforementioned studies measured non-mediated nucleoside permeation in cells in which nucleoside transport activity may be blocked by NBMPR. It may be possible to evaluate this entry process in cells with nucleoside transport systems of low NBMPR sensitivity, such as those used in the present study, in the presence of high concentrations (0.5-1.0 mM) of dilazep (Paterson *et al.*, 1984) or of sulfhydryl reagents (Belt and Noel, 1985).

a. Inhibition of Adenosine Transport in Cells of Hepatomas 3924A and H-35

Transport of adenosine in both hepatoma 3924A and H-35 cells was largely unhindered by concentrations of NBMPR that reduced transport by 50% in S49 cells (Figures 7 and 15). Unlike the nucleoside transport systems in S49 cells and human erythrocytes, those in hepatoma 3924A and H-35 cells were only fractionally inhibited by micromolar concentrations of NBMPR. As well, the nucleoside transport systems of 3924A and H-35 cells were distinctly less sensitive to dilazep and dipyridamole than that of S49 cells (Figures 7 and 15, Table 2). It may be noted in Table 2 that the nucleoside transport system of Walker 256 cells is also relatively insensitive to these inhibitors.

Adenosine uptake in rat tissues has been found less sensitive to inhibition by dipyridamole, than in several other species (Kolassa *et al.*, 1978; Kolassa and Pfleger, 1975; Williams *et al.*, 1984). In guinea pig and rat cardiac tissues, dipyridamole was a weak inhibitor of adenosine

Table 2. Inhibition of nucleoside transport in cultured cells.[1]

Cell type	Inhibitor	IC_{50} (μM)[1]
3924A	NBMPR	1
	dilazep	10
	dipyridamole	5
H-35	NBMPR	5
	dilazep	10
	dipyridamole	0.9
S49	NBMPR	0.001
	dilazep	0.01*
	dipyridamole	0.3*
Walker 256	NBMPR	10
	dilazep	50*
	dipyridamole	5*

[1] The data for 3924A and H-35 cells were obtained in the present study and those for S49 and Walker 256 cells were reported by Gati *et al.*, 1986.

[2] The nucleoside permeant was 20 μM ^{3}H-adenosine except in the instances designated by asterisks (*) in which 100 μM ^{3}H-uridine was employed as the permeant.

uptake (Hopkins and Goldie, 1971; Kolassa *et al.*, 1978; Barker and Clanachan, 1982) and adenosine uptake in rat cortical slices was relatively insensitive to dipyridamole (Davies and Hambley, 1983). The efficacy of a test substance in inhibiting NBMPR binding has been regarded as a measure of the potency of the nucleoside transport inhibitory activity of the substance in cells with nucleoside transporters of high NBMPR sensitivity (Hammond and Clanachan, 1984). Using this criterion, Shi *et al.* (1984), found that dipyridamole was a weaker inhibitor of NBMPR binding in lung membranes from rats than in similar preparations from guinea pigs. The above observations are evidence that the nucleoside transport systems in rat cells are less susceptible to inhibition by NBMPR, dipyridamole and dilazep than those in several other species.

b. NBMPR Binding in Hepatoma 3924A and H-35 Cells

The failure of NBMPR to protect hepatoma 3924A cells against tubercidin toxicity (Figures 3 and 4) was not due to a lack of binding sites for the ligand, as in the instance of Walker 256 cells. Under equilibrium conditions, sites that bound NBMPR with high affinity were present on both hepatoma 3924A and H-35 cells (Figures 8 - 14). Mass law analysis of the binding data from Figures 9, 11 and 13 yielded Kd values for NBMPR dissociation from the NBMPR-binding site complex between 1.2 and 2.1 nM. For H-35 cells, Kd values for site-bound NBMPR were similar to those for 3924A cells, as seen in Figures 17 and 19, which report Kd values of 2.6 and 0.7 nM, respectively, for NBMPR. Figures 9 and 17 indicate that the maximum numbers of NBMPR binding sites (Bmax) present on the hepatoma cells were 2.9×10^5 and 2×10^5 for hepatoma 3924A and H-35 cells, respectively. Therefore, in contrast to Walker 256 carcinosarcoma cells, hepatoma 3924A and H-35 cells possess high affinity NBMPR binding sites. In cells of the Novikoff UA rat hepatoma, in which the nucleoside transport system of low sensitivity to NBMPR, the presence of high affinity NBMPR binding sites has also been reported (Table 3).

Table 3. Comparison of NBMPR binding data from different cell lines.

Cell line	Kd (nM)	Bmax (sites/cell)
Hepatoma 3924A[1]	1.5	$3x10^5$
H-35[2]	2.6	$2x10^5$
S-49[3]	1.0	$1x10^5$
Novikoff UA[4] (crude membrane preparation)	0.48	$1x10^5$

[1] Values reported are means from 3 experiments.

[2] Single experiment.

[3] Cass *et al.*, 1981.

[4] Gatl *et al.*, 1986.

c. Adenosine, Uridine and Dilazep Inhibition of NBMPR Binding

The site-specific binding of NBMPR by hepatoma 3924A cells was inhibited by both adenosine and uridine. The Ki value for the inhibition of NBMPR binding by adenosine was one fifth of that for that of uridine. Inhibition of NBMPR binding by adenosine was also observed in H-35 cells. These observations are in general agreement with other reports of NBMPR-binding inhibition in different cell types by dilazep, adenosine and uridine. Inhibition of NBMPR binding by physiological substrates of the nucleoside transport system suggests that the NBMPR binding sites in the cultured hepatoma cells are associated with the nucleoside transport system.

The Ki value for the inhibition by adenosine of NBMPR binding in hepatoma 3924A cells is similar to previous reported Ki values for the adenosine inhibition of NBMPR binding in HeLa cells (Dahlig-Harley, 1981). Table 4 summarizes the inhibition constants obtained for adenosine, uridine and dilazep inhibition of NBMPR binding in hepatoma 3924A cells. The finding that adenosine was a more potent inhibitor of NBMPR binding in hepatoma 3924A cells than uridine is consistent with the relative affinities of these two nucleosides for the transporter in facilitated diffusion systems of high NBMPR sensitivity (Lum *et al.*, 1979; Jarvis *et al.*, 1982; Plagemann and Wohlhueter, 1980; Paterson *et al.*, 1985); however, the Ki values for both nucleosides in this inhibition are several-fold higher than the Km values for their inward transport. The present study also showed that NBMPR binding activity was about 1500-fold more sensitive to dilazep inhibition than to adenosine; Wu and Young (1984) obtained similar results. The mechanism by which adenosine and uridine inhibit the binding of NBMPR is not apparent. The possibility that adenosine and uridine, as transporter substrates, could induce conformational changes in the transporter that decrease binding site affinity for NBMPR is difficult to reconcile with the general finding of this study that NBMPR-occupied binding sites (in 3924A and H-35 cells) do not appear to interact with nucleoside transporters. In the nucleoside transporter model of Young and Jarvis (1983), it is proposed that high affinity

Table 4. Ki values obtained for adenosine, uridine, and dilazep inhibition of NBMPR binding in hepatoma 3924A cells[1]

Inhibitor	Ki
Adenosine	180 μM[2]
Uridine	500 μM
Dilazep	0.07μM

[1] Values reported are means from 3 experiments.

[2] The corresponding value for H-35 cells was 112 μM.

NBMPR binding occurs at the substrate binding site on the outer aspect of the transporter and that such binding prevents transporter function. However, with the present demonstration that nucleoside transport systems of low NBMPR sensitivity occur in cells that possess high affinity NBMPR binding sites (hepatomas 3924A, H-35 and Novikoff UA), a limitation in the applicability of the Jarvis-Young model has become apparent. Further, the operation of the facilitated diffusion nucleoside transport system in Walker 256 cells, which lack NBMPR binding sites (Jakobs and Paterson, 1984, Paterson *et al.* , 1983b), demonstrates that the latter are not essential to transporter function.

d. Photoaffinity Labelling Experiments

Photoaffinity ligands have inherent affinity for a binding site, but unlike electrophilic affinity ligands, the association with their respective recognition sites is usually reversible prior to photolysis (Feden *et al.*, 1984). Use of NBMPR as a photoaffinity ligand has provided important insight into the molecular biology of nucleoside transport systems. Rosenblit and Levy (1980) concluded that [^{3}H]8-azidoadenosine photolabelled nucleoside transporter sites in rat adipocytes However, Jarvis and co-workrs[10] have shown in photoaffinity labelling experiments that [^{3}H]8-azidoadenosine probably labels the glucose transporter in human red cell membranes. Therefore, experiments that employ 8-azidoadenosine as a probe of the nucleoside transporter must be interpreted with caution.

The photoaffinity-labelled, NBMPR-binding polypeptides from hepatoma 3924A cell membranes described in this study were of higher Mr than those from S49 mouse lymphoma cells (Wu *et al.*, 1983) (Table 5). NBMPR-binding polypeptides of Mr 72,000 - 80,000 were photolabelled in studies with Novikoff UA rat hepatoma cells by (Gati *et al.*, 1986). The nucleoside transport system of Novikoff UA cells, like those of hepatoma 3924A and H-35

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[10] Jarvis, S. M., Young, J. D., Wu, J-S. R., Belt, J. A., and Paterson, A. R. P., unpublished results.

Table 5. Comparison of Mr values of the NBMPR-binding polypeptide from different cell lines.

Cell line	Mr
Hepatoma 3924A	80,000-100,000
H-35	62,000-68,000
S49†	45,000-66,000
AE_1†	Not detectable
Human erythrocytes•	45,000-66,000

† From Young *et al.* (1984)
• From Wu *et al.* (1983)

cells, is relatively insensitive to NBMPR inhibition of nucleoside transport. The NBMPR-binding polypeptide from H-35 cell membranes co-migrated with a 66,000 Mr protein standard on electropherograms. Thus, the relative molecular weights of NBMPR binding proteins from the above-mentioned rat hepatomas are higher than those of several other cell types.

e. Host-Protection Experiments

The presence of a nucleoside transport system of low NBMPR sensitivity in cultured hepatoma 3924A cells was the basis for our choice of the transplantable line of this neoplasm as a tumor model in which to test the host-protection tactic in chemotherapy with tubercidin. The transplantable tumor originated in a male ACI/N rat fed a diet containing N2,N2-fluorenyldiacetamide (Morris and Wagner, 1968). This is a rapidly growing, poorly differentiated tumor.

Protection against tubercidin toxicity in normal ACI/N rats was achieved by administering protective doses of NBMPR-P 1 min prior to toxic doses of tubercidin. Figure 22 shows that the LD_{50}, for tubercidin was increased 2-fold by the prior administration of NBMPR-P (20 mg/kg). The modulation of the toxicity of tubercidin by NBMPR-P, probably derives from reduction of tubercidin entry into vital host tissues. Lynch *et al.* (1981b) were successful in protecting mice against the lethal effects of tubercidin, nebularine and toyocamycin by the same tactic.

In the single chemotherapy experiment undertaken in this study, the i.p. administration of the tubercidin-NBMPR-P combination did not enhance survival time of the tumor-bearing rats. It may be that treatment of the subcutaneous tumor with these agents by the i.p. route is impractical because the agents are cleared from the circulation by the liver before reaching the tumor target. In future attempts to use this neoplasm as a model system in which to test

therapy effects of the tubercidin-NBMPR-P combination, other routes (i.v., s.c.) of drug administration will be employed, as will other sites (i.p., i.m.) of tumor implantation.

f. Conclusions and Future Studies

This study demonstrated that (i) high affinity NBMPR binding polypeptides are present in hepatoma 3924A and H-35 cells, both of which possess adenosine transport systems of low NBMPR sensitivity, and (ii) the NBMPR-binding polypeptides from three hepatomas migrated on gel electropherograms with distinctive Mr values. The former finding implies that, in some cell types at least, polypeptides that bind NBMPR with high affinity are independent of nucleoside transporter elements, and the latter function independently of the former in several cell lines.

It will be important to determine whether the low NBMPR sensitivity of the nucleoside transport systems seen in the three carcinogen-induced hepatoma lines discussed here, is a species-related character, or is a general property of hepatomas. If this were a characteristic of hepatoma cells, and, if nucleoside permeation in normal hepatic cells were largely of high sensitivity to NBMPR, it is conceivable that these characteristics could be exploited in chemotherapy with cytotoxic nucleosides directed at the liver. Preliminary studies suggest that murine hepatocytes possess a nucleoside transport system of high NBMPR sensitivity (Kolassa and Paterson, 1982; Plagemann and Wohlhueter, 1985). Primary hepatocellular carcinoma is an apparent, frequent consequence of chronic hepatitis B viral infections in humans, although this correlation is still controversial (Ziegler, 1977). Thus, an objective of high priority in the extension of this work should be the characterization of nucleoside transport systems of human hepatocellular carcinoma cells, particularly those producing hepatitis B antigens. A number of such cell lines have been established in culture (Macnab *et al.*, 1976; Skelly *et al.*, 1979; Marion *et al.*, 1979).

While a high affinity transport system for adenosine is present in hepatoma 3924A cells, as

are NBMPR binding polypeptides, relationships between the sites at which NBMPR binding occurs and the nucleoside permeation sites are unclear. The evidence presented here that the NBMPR-binding polypeptides are transporter-associated is suggestive only. Genetic techniques may help to determine if the NBMPR-binding polypeptides in cells with transporters of low NBMPR sensitivity are part of the nucleoside transport system. Thus, chemical mutagenization of hepatoma 3924A cells followed by selection of mutants that lack nucleoside transport capability might allow one to determine if loss of NBMPR-binding site activity paralleled mutational loss of transport capability.

This presentation has assumed that NBMPR-binding sites in the cultured hepatoma cells are located on the outer aspect of the cell surface, as in erythrocytes. However, the data presented would not preclude an internal location for the binding proteins because (i) the binding experiments with hepatoma 3924A and H-35 cells were performed under equilibrium binding conditions, and (ii) because NBMPR is lipophilic, it is possible that binding sites for the ligand could be located on the intracellular side of the plasma membrane.

It may be possible to test this possibility with NBMPR-macromolecule adducts (for example, an NBMPR-albumin adduct has been synthesized in this laboratory), providing that the latter interact with NBMPR binding sites. As well, comparison of binding kinetics of NBMPR in cells with nucleoside transporters of high and low NBMPR sensitivity may give some indication of the cellular location of the binding sites.

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